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GROWTH

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GROWTH AS A PROBLEM OF CHEMICAL CATALYSIS

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A. INTRODUCTION

When a chemist, whose interests are not too closely related to Biology or its border line disciplines, is confronted with the problem of growth, one of the first analogies that are likely to come to his mind is that of the "growth" of crystals. To him, the spontaneous creation of these structural units of a high degree of geometrical or rather sterical regularity and symmetry, which for their formation draw upon the "substrate" of the surrounding mother liquor, bears a striking resemblance to the growth of well-organized cells in a nutrient medium. A further analysis of this analogy, however, is bound to expose its superficial character and its many flaws. Perhaps the most striking of these flaws are (*a*) that the molecules, forming the building blocks of a crystal, are molded into the lattice under the influence of Van der Waals forces, and (*b*) that the formation of such molecular lattices does not involve a chemical reaction and, hence, no *chemical catalysis*. For it is precisely this catalytic force which, to the reviewer, would seem to be the logical, common denominator in any attempt at equating the multitude of phenomena which the student of growth in its various aspects is bound to encounter. This concept permits one to trace many features of biological growth processes more or less directly back to fundamental chemical reactions. In this survey, it is proposed to travel in the opposite direction, i.e., to start with a brief exposition of some factors governing the growth of certain large molecules and to proceed from there to more complex phenomena such as the reproduction of macromolecules and virus particles.

B. ON THE GROWTH OF MOLECULES

The molecules which shall interest us here are not of the "ordinary" variety which form the bulk and substance of our texts on inorganic and organic chemistry. They belong to a class which during recent years have been tagged "macromolecules" or, still more impressively, "megamolecules." If their only claim to prominence were their large size, we might well dismiss them as the products of an age which strives to make "bigger and better" things. But they are indeed of

special interest to the student of living matter. Some of them, viz., the proteins and polymer carbohydrates, are the most important ingredients of the material basis of life itself—*protoplasm*. Others have sprung from the chemist's test tubes, e.g., the synthetic resins or rubbers. All of them have certain basic features in common: they represent molecules of a higher order in that they are constructed of many small, simple molecules or building blocks which are forged into long main valency chains, which may be straight or branched. The chains, in turn, may form parcels of well-defined cross-sections by means of cross links or Van der Waals forces. The chains arise through polymerization or condensation reactions, depending on whether double bonds in the original building blocks "unfold" to cement one monomer to the other or whether terminal groups react with one another to form chain links of the type of the peptide bond (CO-NH) with the expulsion of small molecules such as water or HCl. Although many of these chain-forming processes are exothermal and hence voluntary reactions, they have to be initiated by primary steps or trigger reactions where the inertia of the system is overcome by the supply or the lowering of the critical increment or energy of activation. This is the reason why all of these processes need a spark supplied by photochemical energy or the assistance of specific catalysts which have been variously pictured as skilful guides through mountain passes or as lubricants for jammed gears. Those who study growth in the narrow biological sense of the word, i.e., the increase in mass and number of cells of living organisms, are prone to take the cell and protoplasm for granted and to restrict their attention to an analysis of the factors which will affect (in a positive or negative sense) the further fate of these biological units. But there is reason to believe that an extension of this interest to the factors which govern the chemical growth of the macromolecules, which in turn are the substrate of biological growth, may pay dividends in the form of a better understanding of many biological phenomena which are too complex to lend themselves to a more direct approach.

1. *Synthetic Polymers*

The polyacrylic, polystyryl, or polyvinyl resins are, in some respects, greatly simplified models for the protein molecule. This follows from a comparison of a few of their properties, indicated in Table 1.

The mechanism of the formation and degradation of long chain

TABLE 1

	Synthetic polymers	Proteins
Number of different building blocks	One or two monomers	Up to 25 amino acids
Minimum chain length	Two links	280 residues
Structure	Usually straight chains	Folded chains or cyclol patterns
In vitro synthesis	Readily accomplished	Not yet achieved
Catalysts required	Simple peroxides, etc.	Enzymes of unknown structure
Detailed structure	Known	Unknown
Stability	Very stable	Very labile

molecules has been ably discussed by H. Mark in his recent monograph (1) on the basis of the work of Carothers, Marvel, Burk, of Mark himself and others. Let us follow the main lines of his exposition.

If we consider the formation of a long chain molecule from an ethylene derivative as an example of a polymerization reaction, the process may be described by the empirical equation

$$n \text{ times } (CH_2{=}CHX) \rightarrow (CH_2 \,.\, CHX)_n$$

On the left side we have n small molecules endowed with the reactive ethylene double bond and moving independently of each other in the medium. X stands for some substituent such as C_6H_5, OH, OCH_3 or Cl. On the right side is the summary formula for the macromolecule resulting from the reaction of from 100 to 1000 molecules of the monomeric substance. This giant molecule contains all of the atoms of the n original small molecules, linked together by main valences. Assuming that we are dealing with a straight-chain structure, the above equation may be rewritten as follows

$$n \,.\, (CH_2{=}CHX) \rightarrow CH_2{=}CX{-}CH_2{-}CHX{-} \,.\,.\,.\,.\, CH_2{-}CH_2X.$$

One of the salient features of this picture is the change from the unsaturated character of the monomer to the saturated character of the polymer. The lone terminal $CH_2{=}$ residue could hardly be detected with analytical methods in a chain which may comprise as many as 1000 saturated links.

From what we know about the mechanism and kinetics of simple chemical reactions and about the rarity (small degree of probability) of even ternary collisions, we must rule out the idea that all of the n elements shown on the left side of the equation will react simulta-

neously to give rise to the long chain molecule. We have to visualize a growth-like process in the course of which the ultimate, long chain is gradually built up from the individual components. This applies with equal force to the naturally occurring chain polymers such as rubber, cellulose, proteins, etc. In the quantitative interpretation of the mechanism and kinetics of such polymerization reactions a concept originally derived from the study of gas reactions has been most helpful. It is called the ***chain reaction*** theory, not because it leads with necessity to the production of chain-like molecules but because it consists of a chain of elementary chemical reactions which follow each other in rapid succession.

The classical example of a chain reaction is the hydrogen-chlorine reaction which may be initiated by a flash of light. Once started by an intense photochemical impulse the reaction goes to completion with explosive rate. Nernst and Bodenstein formulate it as follows:

$$Cl_2 \xrightarrow{\lambda} Cl+Cl \quad \ldots\ldots \text{ Chain Starter Reaction}$$
$$Cl+H_2=HCl+H \quad \ldots\ldots \text{ First Chain Link}$$
$$H+Cl_2=HCl+Cl \quad \ldots\ldots \text{ Second Chain Link}$$

The "trigger" reaction consists in the absorption of a quantum of light by a chlorine molecule. The energy is used for the dissociation into free halogem atoms which, upon collision with a hydrogen molecule, will in turn give rise to the formation of one HCl molecule and a free hydrogen atom. The latter reacts with a fresh chlorine molecule to yield another HCl molecule and a free chlorine atom. The chlorine atom will now continue the reaction chain in alternation with free hydrogen atoms. The chain is ultimately terminated by one of the following chain breaking reactions:

$$H+H=H_2$$
$$\text{or } Cl+Cl=Cl_2$$
$$\text{or } H+Cl=HCl.$$

The most important feature of chain reactions is that the direct participation of the photochemical impulse or of the catalyst is limited to the starter process. The chain, once started, runs on until it is stopped by the collision of two radicals or energy-rich atoms or by a more or less specific chain-breaker, called an inhibitor. Sometimes the wall of the reaction vessel can act in this manner. We can now understand the great discrepancy between the amount of the energy which was initially supplied to the system and the number of product

molecules formed. In the instance of the chlorine-hydrogen chain 100,000 HCl molecules may be formed as the result of the absorption of a single light quantum. There are strong indications that some important enzyme reactions occurring in the living cell may follow a chain reaction pattern.

How can this concept be applied to polymerization processes? The start of the chain may be visualized as the formation of a "reactive nucleus" by the collision of monomer particles of greater than average energy content ("hot molecules"), by the absorption of light quanta, or by an act of heterogeneous or homogeneous catalysis:

$$CH_2{=}CHX + CH_2{=}CHX \rightarrow {-}CH_2{-}CHX{-}CH_2{-}CHX{-} \quad \text{Collision}$$
$$CH_2{=}CHX + h\nu \rightarrow {-}CH_2{-}CHX{-} \text{ or } (CH_2{=}CHX)^* \quad \text{Photochemical}$$
$$CH_2{=}CHX + M \rightarrow {-}CH_2{-}CHX{-} \text{ or } (CH_2{=}CHX)^* \text{ or } (CH_2{=}CHX)M \quad \text{Catalytic}$$

M stands for any kind of catalyst, the star (*) for an energy-rich or reactive molecule. Since nucleus formation requires a high energy of activation, it occurs only infrequently and, hence, the start of a reaction chain is usually a slow process. The *growth* of the polymer molecule, once it has started, has much in common with an avalanche. The nucleus, by virtue of its high reactivity, picks up other monomeric molecules whenever it happens to collide with them. This process occurs with the liberation of a considerable amount of energy and the reactivity of the nucleus or the free valence at the end of the product of growth is promptly regenerated after the addition of a new group. In other words, we have to postulate that a normal individual act of growth does not impair the reactive capacity of the growing chain. The modest energy requirements of the growth reaction render it a much more rapid process than the start of a chain and favor, consequently, the formation of long chain molecules.

How does the growth of a chain stop? On the basis of the foregoing one could imagine that a chain, once started, might well go on growing until the pool of available substrate is exhausted. Actually many factors tend to "stunt" the growth of the chain molecule. Thus, the migration of a hydrogen atom along the chain or the jump of an atom during a collision may saturate the free valence at the end of the chain; or many-membered rings might be formed, where the head of the chain bites into its tail; or foreign contaminants, e.g., oxygen, might intervene and terminate the chain. From the kinetics of the polymerization process, as it is observed experimentally, inferences may be

drawn concerning the relative and absolute rates of the three fundamental reactions, viz., the starter, the chain propagation, and the chain terminating component of the over-all reaction. It is possible to secure direct information on the average period of growth or the life of the individual chains. It can be shown that the distribution of chain lengths in the total system and the growth period of the separate chains are related intimately to the development of the nuclei, i.e., to the ratio of nucleus formation and rate of chain growth. All of these relationships may be expressed in precise mathematical terms—but these will not be given at this time. It may suffice here to state that the growth reaction of these giant molecules is generally to be regarded as bimolecular, since, owing to its small critical increment, the majority of the collisions between the monomeric molecules and the activated nuclei may well be successful. The kinetic principles of the chain rupture or growth limiting steps are still largely obscure. The kinetics of the various chain molecule formations is as varied as their chemistry. The reactive nucleus which propagates the reaction may differ in nature from case to case. In some instances, e.g., the polymerization of styrene, the intermediate is best formulated as a free radical. It is very short-lived, is produced by the starter reaction, grows rapidly into fairly long chains and the latter are stabilized by a chain breaking step which is not fully understood as yet. The entire life cycle of a chain formation is compressed into the space of 1/100 sec. or less—an interval against which the life cycle of the prodigal "one day fly" appears as eternity itself. The normal polymerization of methylmethacrylate ("Lucite"), on the other hand, follows another course. Here the lifetime of the intermediate products is very long, much longer than would be possible for a free radical of this size. The chain seems to grow in this instance by constantly reproducing the double bond of the original molecule at the end of the chain as follows:

$$CH_2{=}CHX + CH_2{=}CHX \rightarrow CH_3{-}CHX{-}CH{=}\overset{*}{C}HX$$

$$CH_3{-}CHX{-}CH{=}\overset{*}{C}HX + CH_2{=}CHX \rightarrow$$

$$CH_3{-}CHX{-}CH_2{-}CHX{-}CH{=}\overset{*}{C}HX,$$

and so forth.

The formation of the long chain plastic resin from the monomer is controlled catalytically, in this instance, as follows. The monomer, methylmethacrylate, represents a mobile fluid which is marketed

with the admixture of an inhibitor (=negative catalyst) which prevents spontaneous polymerization. After the inhibitor has been removed by extraction with alkali, polymerization may be induced either by heating the monomeric liquid on the steam bath for some time or by adding a suitable catalyst at room temperature, e.g., benzoyl peroxide. Once the polymerization has started, it proceeds until the entire mass becomes the transparent solid known as Lucite or Plexiglas. The polymerization as a strongly exothermal reaction is likely to lead to local overheating and formation of gas bubbles which interfere with the use of the plastic for optical purposes. If, however, certain substances, such as oil of turpentine, are added in small amounts the reaction proceeds more uniformly and a perfectly homogeneous plastic material results. In the instance of the polymerization of acrylic acid, the peroxide-catalyzed process may be arrested at any intermediate stage by simply diluting the system with water and thereby decreasing the probability of collisions between active nuclei and monomeric molecules.

When we compare the vinyl polymers with the polypeptide backbone chain of the proteins, two distinct differences emerge. First, most proteins are built up of many different amino-acid building blocks, whereas the vinyl polymer chain is formed by the lining up of only one kind of monomer. Second, the connecting link in the vinyl chain is a carbon-to-carbon bond, while the peptide bond, –CO–NH–, cements together the amino-acid residues in proteins. It can easily be shown, however, that these differences are not fundamental in nature. For the technically so valuable *copolymers* are produced by the polymerization of mixtures of different monomers, leading to the formation of mixed polymer chains. The well-known vinilyte plastics and the synthetic Buna *S* rubber are examples of such copolymers (see Figure 1).

Even the peptide bond cannot be relied upon as a differentiating feature, since the –CO–NH– linkage is an integral part of Carothers' condensation polymer, Nylon (see Figure 1).

2. *Synthesis of Polypeptides and Proteins*

This brings us to a brief discussion of the formation of polypeptide chains and proteins and of the catalysts involved.

In contrast to the synthetic polymers mentioned above, amino-acids show hardly any tendency to condense spontaneously or under the influence of simple catalysts or at higher temperatures to form pep-

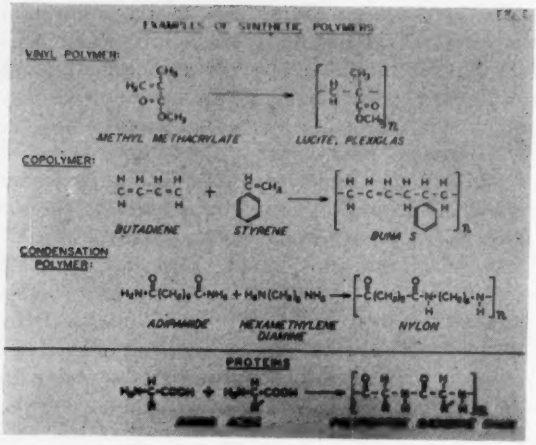


FIGURE 1

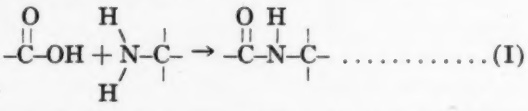
tide bonds and linear polypeptide chains. It is the consensus of opinion (though not definitely established) that the peptides and proteins are synthesized in the living cell by the same enzymes which have been found to hydrolyze peptide bonds *in vitro* in a specific manner and which we call *proteases* as a class. While there is available much experimental information about the resolution of peptide bonds in a variety of configurations by proteolytic enzymes, data concerning their synthetic action are very meager indeed (2). Peptides are usually prepared synthetically by purely chemical methods, involving the reaction of amino-acid derivatives, e.g., the well-known carbobenzoxy-method of Bergmann and Zervas. There is one fairly recent report (3) that an intestinal erepsin preparation promoted the synthesis of dipeptides of histidine, alanine, and arginine to an extent of 30 per cent, provided that the enzyme concentration was kept low. But, in general, the equilibrium between a peptide and its component amino-acids in the presence of a suitable enzyme is situated so much in favor of the hydrolysis products of the reaction that an appreciable reversal of the process may be effected only if the effective concentration of the peptide is held throughout at a level much below its equilibrium concentration (cf. 2). In the instance of the enzymatic synthesis of benzoyl-leucyl-leucine anilide from benzoyl-leucine and leucine anilide, in the presence of papain at pH5, this was achieved through the in-

solubility of the synthetic product. The enzymatic synthesis of acetyl phenylalanyl-glycyl-glycyl-leucine from acetyl phenylalanyl-glycine and glycyl-leucine is made possible by the expedient of splitting the terminal peptide linkage of the acetyl tetrapeptide with the release of leucine as fast as some of the synthetic product is formed, thereby removing it from the reacting system. There is room for considerable doubt as to whether the enzymatic synthesis of any higher polypeptides or proteins has even been demonstrated *in vitro*. It is true that in hydrolysates of certain proteins and in the presence of pepsin, at a pH considerably higher than its activity optimum for hydrolysis, the number of free amino and carboxyl groups will decrease and an insoluble material, "plastein," will form as Borsook and Wasteneys have observed. But it is a moot question as to whether the plastein actually represents a synthetic material of protein-like structure. We know that it cannot be identical with the protein from which the hydrolysate was originally prepared. Thus, the plastein obtained from insulin split products is devoid of any hormone activity (Scott). The plasteins may be denatured proteins or simply conglomerates, lacking any well-defined configuration, in which some of the originally free amino groups have been joined with terminal carboxyl groups in a haphazard fashion (4).

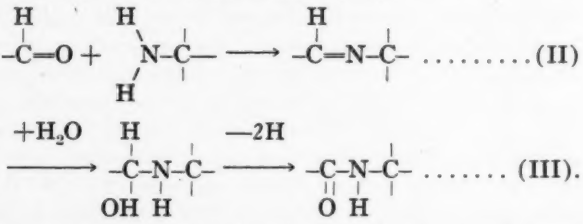
According to our newer knowledge of protein structure, the various amino-acids, contained in a given protein, are not only arranged in a well-defined and reproducible manner in long peptide chains, where the individual residues appear in a definite frequency ratio and in self-repeating unit patterns, but in the native protein molecule these peptide chains are further folded or else arranged in some other geometrically defined fashion, e.g., "cyclol fabrics" (Wrinch). The enzymatic synthesis of protein molecules *in vivo* is obviously such a complex process and the fine structure of even the simplest protein is as yet so incompletely understood, that any attempt at synthesizing a protein from its amino-acids *in vitro* at this stage requires a good deal of optimism. The problem is not so hopeless if it is desired to change one form of a given protein into another, e.g., a denatured into the native form, or to effect relatively small changes in the spatial arrangement of the terminal portion of a protein. In fact, the recent work of Pauling (see below) indicates that such alterations, which probably concern primarily the manner of *folding* of the peptide chain, are today in the realm of possibility.

In a discussion of protein synthesis, the *energy* aspect of the prob-

lem cannot be neglected. Since the hydrolysis of peptide bonds, like all hydrolytic reactions, is mildly exothermal and therefore a voluntary process once the energy of activation has been supplied to the system, the reversal of the reaction, i.e., the synthesis of peptide bonds, requires a driving force. It is probable that the living cell supplies this driving force by coupling protein synthesis with some oxidative process such as cellular respiration or one of its component steps. Voegtlin's observation (5) that the autolysis of muscle and tumor tissue proteins is favored under anaerobic conditions while the presence of oxygen promotes protein synthesis, supports this hypothesis. The mechanism by which the oxidative energy is transferred to the proteolytic system, and how it is utilized, is obscure; attempts to bring about protein synthesis in homogeneous, cell-free preparations will in all probability have to await the solution of this problem. An interesting but definitely speculative schema of a coupling of peptide synthesis with an oxidative, energy-yielding reaction has recently been proposed by Linderstrøm-Lang (6) and Kalckar (7). According to this hypothesis, the peptide bond would not be formed directly from a carboxyl and an amino group (I),



but from a carbonyl and an amino group (II) with the subsequent oxidation of the imide bond to a peptide bond (III):



An advantage of this schema is that the formation of an imide bond requires little or no energy. The formation of the peptide bond by the oxidation of the imide to the CO–NH bond represents a dehydrogenation which would yield more than sufficient energy to cover the requirements of the over-all process. This schema bears a certain resemblance to those intermediate reactions in glycolysis, oxidative

decarboxylation, etc., where oxidative energy is utilized for the synthesis of so-called "energy-rich" phosphate bonds. We shall have more to say about these storage batteries of quick energy in Section *D*.

C. On the Reproduction of Molecules

Any consideration of the problems involved in the synthesis of protein and other macromolecules must, per force, bring up the question of the *reproduction* of such large units. This problem, while it includes the problem of synthesis, is even more perplexing and certainly more complex, since it involves the almost human attributes of "memory," "copying from models," or "manufacture to specifications." While we can readily appreciate—though not always understand—that a living cell manages to synthesize a large variety of small and large molecules with the aid of specific enzymes, it taxes our imagination to visualize the mechanism whereby the introduction of a single macromolecular particle into the cell, such as a virus or bacteriophage unit, causes its multiplication or reproduction to such an extent that the cell perishes under the impact of the very molecules which it modeled from the pattern submitted to it. In the instance of the tobacco mosaic virus it has been shown (8) that the "infection" of a leaf with one or few virus protein particles leads to the synthesis of about 400 mgm. of virus protein by 200 gm. tissue, an amount which represents about 70 per cent of the total protein content of the infected plant material. The production of specific antibodies by the animal organism upon injection of minute quantities of suitable antigens and the reproduction of chromosomes (or genes) by dividing cells represent similar but not quite identical phenomena. In the former case the proteins, formed by the organism upon immunization, are not identical with the introduced proteins (antigens) but *complementary* in such a manner as to form well-defined complexes with them, and in the latter case the reproduction of genes is normally confined to the synthesis of *one* replica per nucleus or cell.

1. *Enzyme Formation from Precursors; Autocatalysis*

In their desire to arrive at a rational explanation of the reproduction of macromolecules by the cell the microbiologists and cytologists have scanned the files of the biochemists for observations of a similar but better understood character and they were struck by the apparent analogy existing between the reproduction of macromolecules by living cells and the formation of enzymes from inactive precursors which

is a special case of *autocatalysis*. The latter term applies to catalyzed reactions in the course of which increasing amounts of new catalyst are produced, thereby leading to a progressively accelerated rate of reaction. Such instances of autocatalysis may be recognized by the characteristic shape of the time-rate curve of the reaction which is bent upwards, i.e., shows a slope becoming steeper with time.

The formation of enzymes from their inactive precursors has been studied in detail by Northrop (9) and his associates. Freshly secreted pancreatic juice is devoid of proteolytic activity. Upon standing, or the addition of an activator secreted by the small intestine (enterokinase), or of a small amount of active trypsin, trypsin is formed, at a rate increasing with time, from a preformed, inactive precursor (trypsinogen). If one works with highly purified substances, preferably after repeated recrystallization, an autocatalytic reaction curve is obtained in experiments of this kind. It is not possible to postulate *one* mechanism of formation of enzymes from their inactive precursors which would be applicable to *all* cases since the phenomena observed differ from case to case. Thus, the transformation of chymo-trypsinogen into chymo-trypsin under the influence of small amounts of trypsin is a catalysis where the rate is proportional to the trypsin and also to the chymo-trypsinogen concentration. While the reaction, e.g., with regard to its dependence on the pH, appears to be analogous to an ordinary protein hydrolysis, the activation of chymo-trypsinogen does not seem to consist in the splitting off of some residue, although an increase in the number of free amino groups per mole is observed. Northrop thinks that the transformation of the inactive into the active form of chymo-trypsin may consist in the opening of a peptide ring. It is interesting to note that the conversion is not catalyzed by chymo-trypsin itself and that, therefore, no autocatalysis is involved here. Quite the contrary is true for the conversion of trypsinogen into trypsin. Here traces of trypsin start off a truly autocatalytic reaction. As in the instance of chymo-trypsin, however, it does not seem that a portion of the molecule is split off during the activation process. It is held probable that the change-over from the inactive protein to the enzyme involve somes internal rearrangement in the structure of the molecule. It should be noted that trypsin is not the only substance capable of bringing about this transformation. The activator enterokinase from the small intestine has already been mentioned. Much discussion has taken place on the question whether enterokinase combines with trypsinogen in a stoichiometrically defined manner to form

the active trypsin complex or whether the kinase is an enzyme catalyzing the trypsinogen-trypsin transformation. The recent work by Kunitz (10) favors distinctly the latter view. It would seem that the conflicting results obtained by various workers were due to the fact that during the reaction an inert protein, as well as the active enzyme, is formed, the relative amounts depending on the acidity of the solution. The entire reaction may be quantitatively described on the assumption that the rate, at which the active enzyme is formed, is a function of the concentration of precursor and of the activator, whereas the rate of formation of the inert protein is proportional to the trypsin and the precursor concentration. The balance between the two reaction rates is determined by the hydrogen ion concentration. Another substance able to convert trypsinogen into active trypsin is a proteolytic enzyme secreted by a mold (Penicillium (11). When the activation is carried out at pH 5, where trypsin itself is inactive, the activation curve is no longer autocatalytic in character but assumes the simple logarithmic shape as we find it for instance in the transformation of chymo-trypsinogen into chymo-trypsin under the influence of trypsin. The trypsin formed in this way seems to be identical in every respect with the one produced by autocatalysis. We have to conclude that all of the activators, trypsin, enterokinase, and the mold kinase, act in the same way and, probably, attack the same peptide bond in the precursor molecule.

Still another type of reaction is presented by the conversion of pepsinogen into pepsin, a reaction recently studied in detail by Herriott (12) after isolating the precursor in crystalline and, presumably, pure form. The activation process at pH 4.65 follows the course of an autocatalysis and is therefore caused by the pepsin itself. In contrast to the activation of trypsinogen or chymo-trypsinogen, about 15 per cent of the peptide nitrogen is split off from the rest of the molecule during the reaction. Inasmuch as the specificity of pepsin is confined to the breaking of peptide bonds contained in certain configurations (Bergmann and Fruton), the formation of active pepsin from pepsinogen must consist in the rupture of one or more peptide bonds in the precursor molecule, somewhere near the end of the polypeptide chain. Another interesting fact that came to light in Herriott's work is that the configuration responsible for the immunological species-specificity of pepsin must reside in the precursor molecule. For, if swine pepsinogen is activated by chicken pepsin, swine pepsinogen is formed (13).

Northrop (9), in discussing the bearing of the experiments on the

formation of enzymes from inactive precursors on the problem of virus and phage reproduction by living cells, expresses his views as follows:

> These reactions in some cases are autocatalytic and therefore present a formal analogy with the growth of cells and the increase in viruses. There is an essential difference between the growth of cells and the formation of enzymes in that cells can use energy to synthesize themselves from simpler compounds whereas, so far as is known, enzymes cannot. It is not yet known whether phage or other viruses possess this power of synthesis. Until this knowledge is obtained it does not seem possible to decide as to which of the two analogies has a real physical basis and which is merely formal. In the meantime it appears to the writer that the much more simple analogy with enzyme formation has predicted the facts better than the analogy with cell growth. Thus, some viruses and phages, at least, are proteins and may be isolated by the methods of enzyme chemistry; they may be inactivated and reactivated; they have no measurable respiration. Pepsin and trypsin are immunologically distinct from their precursors, just as phage and virus are immunologically distinct from their host cells.
>
> The curves for the rate of formation of phage and of enzymes by cells or from their precursors are similar and more or less autocatalytic in character.
>
> It is evident from the foregoing that a formal analogy exists between the production of phage and the formation of enzymes.

An adoption of the view that a close analogy exists between the formation of proteolytic enzymes from their inactive precursors and the mode of reproduction of viruses and bacteriophages would seem to shift the emphasis from the virus to the precursor problem. It would imply that there exists in every susceptible cell a store of "prefabricated" virus or phage in the form of the precursor molecules which would then be released "from stock" upon the entry of one or few virus molecules. Since some cells, e.g., the chorioallantois membrane of the chick embryo, are susceptible to a great variety of viruses, ranging all the way from equine encephalomyelitis to vaccinia elementary bodies, this view would imply also that this tissue contains huge numbers of the proper precursor molecules for each and everyone of the many viruses for which it is susceptible. If the relationship between the virus and its precursor is chemically as close as that between trypsin and trypsinogen for example, then this view is obviously highly improbable. It would mean, at best, the substitution of one unknown, the precursor, for another unknown, the virus synthesis.

Delbrück (14) has recently advanced an interesting hypothesis on the autocatalytic synthesis of polypeptides, with particular reference

to the problem of chromosome reproduction. The most stringent condition in this case is that such a hypothesis must not only explain how a chromosome or a gene reproduces itself but also why in the normal cell the reproduction is limited to the production of just *one* replica. The hypothesis starts out from the hypothetical schema for the formation of the peptide bond by the oxidation of an intermediary imide bond, as proposed by Linderstrøm-Lang and also Kalckar (see page 10). The oxidation of this bond may be formulated as a dehydrogenation in such a way that two hydrogen atoms are removed from the molecule after the addition of one water molecule (see Equation III on p. 10). In accordance with Michaelis' theory of two-step oxidations, it is to be assumed that the two hydrogen atoms are taken off one by one. The intermediate monohydrocompound would have the structure of a semiquinoid radical with a high energy content. For its oxidation, Delbrück argues, an oxidant of a very positive oxidation-reduction potential would be required which the cell is not likely to possess. The energy of the radical or of its cation could, however, be lowered by the establishment of resonance between two states of equal energy. This could be brought about by complex formation between the cation of the monohydro radical and another molecule containing a peptide bond. This reaction would be highly specific since the catalyst must show a steric fit with the substrate in order to permit sufficiently close contact and since the energy level of the additional electron in the substrate must be similar to that of the same electron in the corresponding state in the catalyst. These requirements could best be met by a catalyst with a chemical structure very similar to that of the product of oxidation, i.e., the finished peptide. This hypothesis could explain why all enzymes thus far found capable of resolving and forming peptide bonds are themselves proteins or polypeptides. This schema could also be used to explain

> why the reproduction of chromosomes is normally confined to the synthesis of one replica. It separates the peptide bond formation into two oxidation steps. The two steps may well be separated in time, in such a manner that one step is first carried out along the entire length of the chromosomes, before the second step is initiated. The intermediate situation would then be a semiquinone double chain, with a negative charge for each prospective peptide bond. It must be supposed that the two steps of oxidation require different redox conditions, so that each can occur only at a particular phase of the cell cycle, and the first step, the formation of the intermediate complex, cannot be repeated until the second oxidation has restored the original condition (Delbrück, 14).

The reviewer holds no brief for or against this novel hypothesis. He is mainly reporting it here to show that the whole problem is still in a very speculative stage.

2. *Polysaccharide Synthesis In Vitro*

Until very recently, all attempts to reverse reactions involving the breakdown of carbohydrates, ***in vitro*** and in cell-free systems, met an invisible barrier at the hexosephosphate stage. Although it was known for a long time that the hexose, forming the substrate of fermentation, was derived from glycogen in the animal cell and from starch in the plant cell, the rebuilding of hexose residues into polysaccharides seemed to many a prerogative of the living cell, much as the synthesis of proteins. When, therefore, Cori, during his address before the Federation Meeting at Toronto in 1940, mixed several clear solutions in front of the audience, placed the test tube in his vest pocket, and after a little while took it out and showed that a turbidity had formed which gave the blue color reaction with iodine typical for starch, he seemed to have performed a miracle. Actually it was the logical sequel of his discovery, made several years before, that the primary product of the breakdown of glycogen by a phosphorylating enzyme (phosphorolysis) is glucose-1-phosphate rather than the stable glucose-6-phosphate into which the labile primary product is rapidly converted by another enzyme. Conversely, in order to be resynthesized to starch or glycogen, glucose must first be phosphorylated to the glucose-1-phosphate. The rest is more or less automatic, since the latter, in presence of phosphorlyase and adenlyic acid, gives rise to glycogen formation. At physiological pH, the equilibrium as catalyzed by the enzyme is about 77 per cent in favor of the glycogen (15). The change in free energy during this reaction is very small, which must mean that the ester linkage in glucose-1-phosphate is energetically nearly equivalent to the glucosidic linkage bonding the hexose residues in the large polysaccharide molecule. The enzyme catalyzing this reversible reaction in muscle extracts, muscle phosphorylase, has lately been isolated in crystalline form (16). While this enzymatic synthesis of a macromolecule is of great importance and interest in itself, its main significance for our present discussion lies in the fact that the synthetic reaction has to be ***primed*** by the addition of small amounts of polysaccharide (17). The nature of the polysaccharide synthesized is determined by the type of phosphorylase rather than by the nature of the polysaccharide used for

priming the reaction. Thus, muscle phosphorylase, when primed with liver glycogen, synthesizes a typical starch *in vitro*, and liver phosphorylase, when primed with plant starch, synthesizes glycogen. The "catalytic effect" of the activating polysaccharide is illustrated in the diagrams reproduced in Figure 2 from a paper by Green and Stumpf

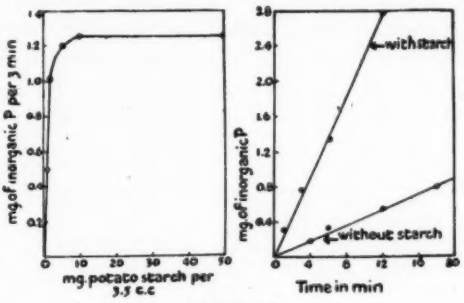


FIGURE 2

(18). From the above facts it follows that one and the same enzyme, muscle phosphorylase, is able to synthesize both starch and glycogen, the former *in vitro* and the latter in the intact muscle cell. And this in spite of the fact that the newer work by Haworth, Bell, and others has shown that the structural plans of glycogen and starch are markedly different, although both polysaccharides are built up of glucose units. While it is permissible to speak of the effect of the priming polysaccharide as catalytic, in the sense that only minute amounts are required and that the concentration of the priming substance is not changed during the reaction [although a thermolabile (phosphorylase) and a thermostable catalyst (adenylic acid) must also be present], the reaction is not an autocatalysis. It could be shown (18) that the polysaccharide formed during the synthetic process cannot be used as primer. The conclusion that the function of the priming carbohydrate cannot be that of a "template" or "matrix" molecule serving as a model for mass production by the enzyme, is further underlined by the shape of the reaction curve which is not that of an autocatalytic process. Whatever the explanation of this phenomenon may be, it is to be expected that its elucidation will contribute materially to our knowledge of the mechanism of the synthesis of polysaccharides by phosphorylases and, perhaps, of the enzymatic synthesis of macromolecules in general.

3. *In Vitro Synthesis of Antibodies*

The feat suggested by the above title, would, a few months ago, have been considered by most immunologisits and biochemists a figment of imagination. That it is today, if not an actuality, a near reality is due to the daring and fresh viewpoint of an outsider in the field of immunochemistry, Linus Pauling of the California Institute of Technology. Less than two years ago, Pauling wrote a paper on *A Theory of the Structure and Process of Formation of Antibodies* (19) which must have struck most of us as highly speculative. It was adorned with drawings, such as those reproduced in Figures 3

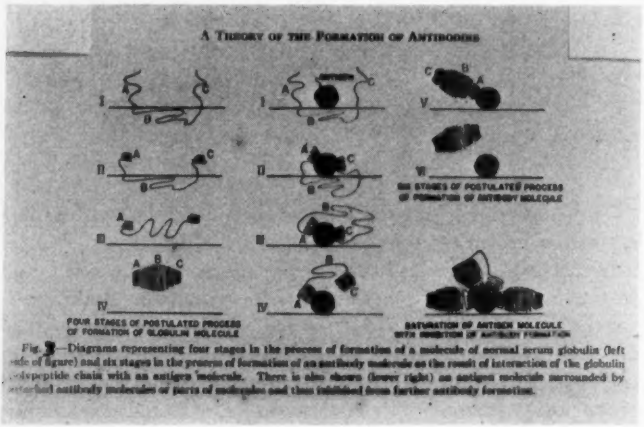

A THEORY OF THE FORMATION OF ANTIBODIES
FOUR STAGES OF POSTULATED PROCESS OF FORMATION OF GLOBULIN MOLECULE
SIX STAGES OF POSTULATED PROCESS OF FORMATION OF ANTIBODY MOLECULE
SATURATION OF ANTIGEN MOLECULE

Fig. 3.—Diagrams representing four stages in the process of formation of a molecule of normal serum globulin (left side of figure) and six stages in the process of formation of an antibody molecule as the result of interaction of the globulin polypeptide chain with an antigen molecule. There is also shown (lower right) an antigen molecule surrounded by attached antibody molecules or parts of molecules and thus inhibited from further antibody formation.

FIGURE 3

and 4, which seemed to be examples of "paper chemistry" brought to the extreme.

The arguments put forward in that remarkable paper were based on the hypothesis, advanced several years previously by Pauling and Mirsky, that the protein molecule in its native state represents a neatly folded polypeptide chain which, upon denaturation, unfolds reversibly. Recent experiments by Cannan, Rothen, and others on the shape of native and denatured ovalbumin appear to support this view. Pauling assumed that

> antibodies differ from normal serum globulin only in the way in which the two end parts of the globulin polypeptide chain are coiled,

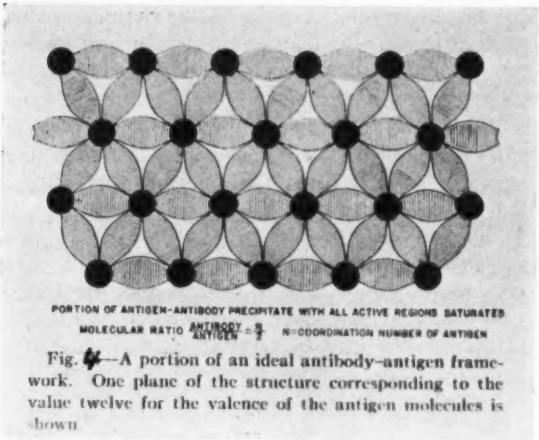

PORTION OF ANTIGEN-ANTIBODY PRECIPITATE WITH ALL ACTIVE REGIONS SATURATED
MOLECULAR RATIO ANTIBODY/ANTIGEN = n/2 n=COORDINATION NUMBER OF ANTIGEN

Fig. 4.—A portion of an ideal antibody-antigen framework. One plane of the structure corresponding to the value twelve for the valence of the antigen molecules is shown.

FIGURE 4

these parts as a result of their amino-acid composition and order having accessible a great many configurations with nearly the same stability; under the influence of an antigen molecule, they assume configurations complementary to surface regions of the antigen, thus forming two active ends. After the freeing of one end and the liberation of the central part of the chain, this part of the chain folds up to form the central part of the antibody molecule with two oppositely directed ends able to attach themselves to two antigen molecules.

Pauling further proposed to test this hypothesis experimentally by reversibly denaturing normal serum globulin, bringing it in contact with an antigen in the test tube, and then slowly reversing the denaturation, thereby giving the antigen a chance to impress its specific surface configuration upon the terminal parts of the globulin chain in a manner similar to the formation of a relief by a suitably shaped matrix or die. I do not believe that this plan was taken very seriously by experts in the field at the time it was proposed. But, as you know, a short while ago Drs. Pauling and Dan Campbell have claimed to have succeeded in experiments of this type. The antigens used by the California workers were (*a*) a protein marked with azo groups and thereby converted into an antigenic substance in accordance with Landsteiner, and (*b*) a *Pneumococcus* polysaccharide. Pending the verification of this work, it might be well to withhold final judgment

and wait before discarding our antibody-manufacturing rabbits, horses, and guinea pigs in favor of the test tube and incubator. Provided that the reports can be confirmed and if one wishes to pin the label of "catalyst" on one of the components of the system, the antigen would be the first in line. In its presence an inactive protein, the denatured serum globulin, is transformed into a protein endowed with specific biologically activity, viz., the corresponding antibody. This brings to mind the claim by Count Schwerin and his associates, put forward 20 years ago, that it is possible to "transfer" diphtheria antitoxin activity from the euglobulin fraction of *horse* serum to the pseudo-globulin fraction of *human* serum *in vitro* by mixing solutions of the two proteins and then precipitating the euglobulin by dialysis against water. It is quite possible that the transfer involved the intermediary, reversible denaturation of one or both proteins. Although the two sets of experiments are admittedly not quite analogous, it is not altogether impossible that they may have a common denominator since both represent the transformation of an inactive protein into a protein with specific immunological activity *in vitro* upon contact with another protein which is closely associated with that particular type of activity. Other forerunners of Pauling are cited in a recent editorial in the A.M.A. Journal (19b).

D. On the Reproduction of Virus Particles

1. *"Molecule" versus "Cell" Concept*

I am sure that most of us will agree that a controversy on the question, whether a virus molecule of the kind of the tobacco mosaic virus nucleoprotein is living or not, is rather meaningless. It all depends on the criteria which one wishes to employ in defining life or vital activity. If evidence of an independent metabolism is demanded before a particle is dignified with the attribute "alive," then the viruses, including the bacterial viruses or phages, have no claim to being so honored since all attempts to demonstrate respiratory or glycolytic activity in a variety of viruses have failed. If, on the other hand, the faculty of multiplication and of "breeding true" is considered as the prerogative of living things, then the viruses have a better chance.

Attempts to determine whether the viruses are simply minute, complete cells or whether they are single, although very large, molecules have decidedly more merit. For example, if they could be recognized as cells their multiplication would be no more mysterious than the multiplication of any other cell. The fact that they can only multiply in

contact with suitable host cells might then be explained by assuming that they are unable to synthesize certain substances of complex structure and that they depend on obtaining these from the host cell. The problem has recently been discussed in some detail by Barbara Holmes and Antoinette Pirie (20) and by Laidlaw (21). It is likely that the class name of filterable viruses embraces all kinds of forms and transitions from complete, living cells to single protein molecules which have become so completely dependent on the host cell that the only attribute of life which they have retained is that of breeding true. There is already available a considerable body of evidence, both from size measurements and from chemical analyses, that viruses range in size from particles not much larger than the hemocyanine molecule to dimensions of the same order as those of certain small bacteria occurring in sewage (22), and that their composition may vary from that of simple nucleoproteins to that of the same kind of complex mixture of proteins, lipids, carbohydrates, etc., as we encounter in protoplasm and whole cells. As a general rule it would seem that plant viruses represent monodisperse, crystallizable nucleoproteins while animal virus preparations are heterodisperse, non-crystallizable populations of particles of relatively large size and highly complex chemical composition. The position of bacterial viruses (bacteriophages) within this system is not yet quite clear, especially since there seem to exist very considerable differences between the various types of phages, at least with regard to their size (23). The question is further complicated by the fact that photomicrographs obtained with the electron microscope of different phages show uniform suspensions of dense, round particles in some cases (24) and spermatozoa-like forms with tails and internal structure of the main body in others (25).

2. *The Virus Precursor Problem*

The precursor problem has been investigated with particular intensity with regard to the occurrence of precursors of bacteriophages in the bacterial host cells. The leading proponent of the view that such precursors do exist is Krueger. References to his work and a critical evaluation of the data on which his conclusions are based will be found in a recent review article by Delbrück (26). Some aspects of this problem and its bearing on the reproduction of virus particles have already been touched upon in an earlier section of this survey (see pp. 11 to 14).

Here, I wish to bring to your attention an interesting observation

made in the course of the study of bacterial lysis by *B. coli* phage with the aid of the electron microscope (27). The electron micrographs, obtained at various stages of the process, reveal that the phage particles are adsorbed on the bacterial surface. The latter then loses its sharply defined outline and bursts, permitting the cytoplasm of the cell to leak out and to leave behind a resistent "ghost" much as is observed in the hemolysis of red blood cells. In some instances there are now observed in and on the "ghost" well-defined bodies of cubic outline which are possibly crystals. Upon continued bombardment with the electron beam of the microscope the phage particles as well as the cubic bodies are "burned" out by the impinging electron stream, transforming the former into ring-shaped or hollow "bags" and the latter into still cubic-shaped crystal "ghosts." The similarity in sensitivity to electron bombardment displayed by the phage particles and the crystals would seem to suggest that they are related in chemical structure. If this is the case then the crystals might possibly be connected with the centres of origin of the phage particle existing in the cell, in other words they may represent the postulated intracellular precursor substances of the phage. Figure 5, showing the burned out

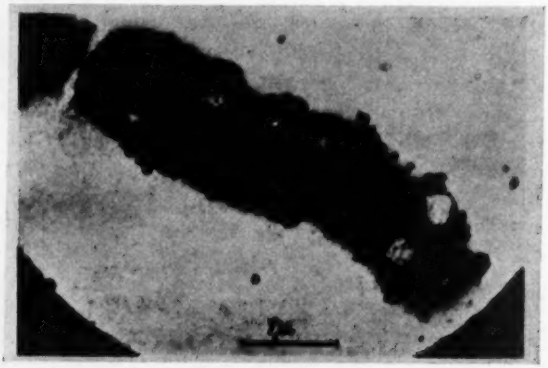


FIGURE 5

crystalline bodies and phage particles in the case of *B. coli,* is taken from the article by Ruska (27). A similar electron micrograph was recently obtained by Dr. Anderson in the RCA Laboratories on one of our Dysentery phage preparations (unpublished work). The unambiguous interpretation of these pictures must be left to the future.

3. *Source of Energy for Virus Reproduction*

This final section of our discussion is distinctly speculative in character. If, for the purposes of argument, we adopt the view that certain viruses are simple nucleoprotein molecules which, when introduced into suitable host cells, start to reproduce themselves in large numbers without possessing a metabolism or energy-yielding reaction mechanism of their own, then it follows that the virus particles are not only parasitic with respect to the building materials which they divert from the normal channels of the cell but that they also prey on the *energy sources* of the cell. How can we visualize the mechanics of such acts of piracy? The question may be formulated more precisely. We know that certain compounds containing phosphate residues in a linkage which Lipmann calls the "energy-rich phosphate bond," such as adenosinetriphosphate or creatinephosphate, serve as sources of quick energy in muscular contraction and in a number of intermediary reactions in glycolysis and other enzymatic processes in the living cell. It had been assumed for some time that the contractile substance of the muscle fiber, the protein myosin, is recharged continuously by utilizing, in some obscure way, the energy stored up in adenosinetriphosphate by means of the glycolytic mechanism. Recent experiments by Engelhardt (28) and his co-workers at the Biochemical Institute of the U.S.S.R. Academy of Sciences in Moscow, for the first time, seem to shed some light on the manner in which the *chemical energy* furnished by the muscle metabolism is transformed into the *mechanical work* of the muscle fibers. The Russian investigators had observed that the enzymatic activity of muscle preparations concerned with the splitting of adenylpyrophosphate into adenosinediphosphate and inorganic phosphate (adenylpyrophosphatase) is closely associated with the muscle protein, myosin. Their findings pointed to the identity of the protein and the enzyme. Subsequently, Needham and his colleagues in Cambridge (29) showed that adenylpyrophosphate alone from many substances tested is capable of abolishing, reversibly, the flow birefringence of myosin solutions. Then Engelhardt (28) reported that synthetic myosin fibers, which in many respects are a model for the native muscle fiber, will extend reversibly when immersed in a solution of adenosinetriphosphate, and that a close parallelism exists between the maintenance of the native state of the protein, its pyrophosphatase activity, and the specific effect of the substrate on the fiber model. It is obvious that these discoveries, if they can be confirmed, would furnish the missing link between the

production of chemical energy and the ability of muscle to perform mechanical work. There is a distinct possibility that the myosin molecule itself, in the course of the reaction with adenylpyrophosphate, undergoes a reversible intermediary phosphorylation and that the introduction of the highly polar phosphate groups into the protein chain is connected with the changes in intramolecular distances which find their macroscopic expression in the extension or relaxation of the myosin fibril. Could it be that a similar reaction mechanism forms the basis for the stealing of energy from the cell by the parasitic virus? This theoretical possibility would assume the status of a probability if it could be shown that virus particles possess the catalytic power required for attacking energy-rich phosphate bonds. It seems significant and indeed suggestive that the *only* type of enzymatic activity thus far encountered in purified virus preparations is the ability to split phosphate ester linkages! Thus, Schüler (30), working in Meyerhof's institute, found that a centrifugally purified *B. coli* phage exhibited intense phosphatase activity; and M. G. MacFarlane and Dolby (31) state that vaccinia elementary bodies as well as two out of three samples of Bushy stunt virus and a single sample of tobacco mosaic virus protein exhibited hydrolytic activity towards several phosphate esters. It is obviously too early to speculate at this point on the detailed mechanism by which the virus contrives to utilize the energy thus "stolen" from the host cell for its reproduction. But I believe you will agree that these recent discoveries open up new perspectives and promising avenues of approach towards the solution of some of the fundamental problems in Biology.

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THE RELATION OF GENETICS TO GROWTH-FACTORS AND HORMONES*

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It is a widely accepted view that growth, development, and differentiation of an organism are dependent on the activity of its genes. (For a recent and comprehensive review and discussion of this general topic, see Wright, 1941.) Genetic control may vary from cases in which the relation between the gene and the observed character is relatively direct to those in which it is complex and consequently difficult to interpret. In many instances a further difficulty in the analysis of the primary effect of individual components of the gene complex arises from the fact that mutations of indispensable genes are often lethal.

Because of these conditions, the physiolgical geneticist has usually concerned himself with the investigation of the action of genes controlling so-called "terminal reactions," that is, those having to do with the production of substances such as anthocyanin and melanin pigments, the presence or absence of which does not vitally concern the life of the organism. There is good reason to believe that the mechanisms by which genes control these non-vital processes are essentially similar to those by which other genes control the indispensable and complex processes of differentiation and development. Investigations such as those on anthocyanin pigments of plants (Lawrence and Price, 1940), and on the melanin pigments of animals (Wright, 1941) have led to the general concept that genes express themselves through the control of specific biochemical reactions. This control can be most simply explained by supposing that genes act directly by determining the specificities of enzymes which in turn control the specific biochemical reactions involved. For example, the work of Winge and Laustsen (1939) on yeast enzymes and Irwin and co-workers (Irwin and Cumley, 1942) on specific blood proteins in birds strongly suggests a direct relation between particular genes and specific enzymes and proteins. On the basis of this general concept an organism can be thought of as resulting from the action and interaction of a complex of specific

*The work on *Neurospora* summarized in this paper is supported by grants from the Rockefeller Foundation and from the Penrose Fund of the American Philosophical Society.

biochemical reactions each of which is controlled through enzymes by specific genes.

To illustrate more specifically our ideas of the nature of gene action, some of the work on eye-pigment development in Drosophila will be reviewed (see Beadle and Tatum, 1941a, and Kikkawa, 1941). In this insect the brown pigment component in the eyes, apparently melanic in nature, is synthesized in a series of step-wise reactions leading from the amino-acid tryptophane, through alpha-oxytryptophane, kynurenine, and *cn*+ hormone to the final pigment. All of these substances except the final pigment are diffusible in the organism and are therefore readily worked with. The chain-reaction interpretation is based on experimental data obtained from eye transplantation experiments, injection of eye and body fluid extracts, isolation of substances, and tests of known chemicals for activity. The sequence of reactions concerned may be blocked in any one of a number of places as a result of inactivation of specific genes. The normal wild type allele of each of these genes is necessary for the corresponding step to take place, and each presumably acts through a specific enzyme which is required for a reaction. This analysis of eye-color development illustrates several points of importance in connection with theories of gene action: (*a*) The expression of a given character is usually the result of a number of biochemical reactions taking place in series or in parallel. (*b*) Each reaction in the series is controlled by a specific gene, probably acting through a specific enzyme. (*c*) If any step is blocked genetically the substance ordinarily acted upon in that step may accumulate. (*d*) If the immediate product of any single blocked step can be supplied artificially, and utilized, the subsequent reactions proceed normally and the character expresses itself as though the step were not blocked.

Since essentially all processes in an organism are biochemical, each should be resolvable into a series of specific reactions. Each reaction presumably is controlled by a specific gene. These gene-controlled reactions should include syntheses of all biologically active substances, both diffusible and non-diffusible. Those of the diffusible category, particularly the ones produced in localized regions, include substances known as hormones. If as a result of mutation an indispensable non-diffusible substance cannot be synthesized, the individual cells of the organism cannot survive in any environment and the gene change responsible is a lethal mutation. If a mutation occurs in an organism living in an environment in which the product of the blocked synthesis

is readily available, the organism will survive in spite of the mutation. In the absence of the specific product, however, such a mutation would be lethal. Such essential diffusible substances are known as growth factors or vitamins.

If all such syntheses are gene controlled, it should be possible to block the production of specific substances in the cell by inducing mutations in the genes controlling these syntheses. We might then expect to find mutations of several classes: (*a*) Lethal mutations in which the syntheses of essential non-diffusible substances are blocked. (*b*) Mutations in which the syntheses of non-essential substances, either diffusible or non-diffusible are blocked, the latter perhaps leading to the development of variant morphological types. (*c*) Mutations characterized by inability of the organism to synthesize essential diffusible substances such as vitamins, growth factors, and amino acids, which could be used by the organism if supplied as such from the environment.

The ascomycete *Neurospora crassa* is suited to an experimental approach to this problem since its genetics is well known through the work of Dodge, Lindegren, and others, and since the nutrient requirements of this mold are relatively simple. We have developed a technique of inducing mutations in this organism by means of x-ray treatment of perithecia or conidia and subsequent isolation of single spore cultures (Beadle and Tatum, 1941b). These cultures are initially supplied with many nutrient materials in the form of yeast and malt extracts ("complete" medium). After the strains have been established on complete medium in this way they are tested for any loss in synthetic ability by transferring them to a medium containing only sugar, inorganic nitrogen, salts, and biotin, these constituting the minimal requirements of the organism. If a strain derived from x-rayed material grows on the complete medium but fails to grow on the "minimal" medium it is presumed to have lost the ability to synthesize some essential substance. Such deficient cultures are then tested on a medium containing a mixture of known vitamins and growth factors and on a medium containing hydrolyzed casein as a source of amino acids. If the strain grows in one of these media, it is tested again on the individual components—vitamins or amino acids as the case may be. By means of this general technique approximately 30,000 cultures from single-spore isolations have been obtained and tested. Of these more than 50, including recurrent mutations, have been found to have lost the ability to synthesize known or unknown

substances. The genetic evidence to be taken up later indicates that many at least of these defects in metabolism are inherited as if they were differentiated from the normal condition by single genes.

Mutants so far obtained include strains deficient in the syntheses of the known vitamins, thiamin, thiazole, nicotinic acid, pantothenic acid, para-aminobenzoic acid, and pyridoxin, and the amino acids methionine, lysine, arginine, leucine, and tryptophane. Strains have also been obtained which appear to require certain purines or pyrimidines, but the requirements for these have not been completely worked out as yet.

In general, growth of these mutant strains is a function of the amount of the required substance provided. The growth can be estimated quantitatively either by determining the weight of mycelia produced in liquid media or by measuring the rate of linear progression of the mycelial frontier along the surface of agar media in a horizontal tube (Beadle and Tatum, 1941b). It has been pointed out elsewhere that it is possible to use such mutant strains for vitamin and amino acid assays.

On theoretical grounds we should expect to find mutant strains deficient in the syntheses of substances whose essential roles have not as yet been recognized. This expectation appears to be justified. For example, one strain requires a substance present in yeast extract and casein which is not replacable by any known vitamin or amino acid so far tested. In collaboration with Dr. D. M. Bonner we are working on the isolation and identification of this substance. Other strains will not grow on known substances that have been tried but require growth factors present in yeast extract or in coconut milk.

In addition to these strains in which the synthesis of essential diffusible substances is blocked by the gene mutation, a considerable number of the x-rayed spores fail to germinate or survive but for a short time on complete medium. Some at least of these failures to germinate or survive may result from induced lethal mutations in which syntheses of essential non-diffusible substances are blocked. Still other mutations have been found which are expressed as abnormal cultural or morphological characters. Certain of these strains grow in compact clumps of various types, others have characteristic abnormal growth forms, and some do not produce conidia. These morphological types may be the result of mutations in genes controlling syntheses of non-diffusible substances which are not essential for growth but which are essential for normal growth form.

As indicated above, genetic analysis of the mutant strains so far tested indicates that they are differentiated from normal by single genes. This is relatively easy to establish since single ascospore isolates of *Neurospora* are haploid and genetically pure. A mutant can therefore be crossed with the normal strain of the other mating type and the eight ascopores recovered from a single ascus. These can be established on complete medium and then tested for the character. If the mutant differs from the normal by a single gene, the character will segregate and will reappear in four of the eight ascospores.

The fact that the mycelium of *Neurospora* is haploid rather than diploid, as are somatic tissues of higher plants and animals, is an advantage in making possible the ready detection of mutations. But this condition is a disadvantage in so far as it makes it impossible to determine by the usual method whether mutant genes are dominant or recessive to their wild type alleles. This is important on theoretical grounds since it is probable that recessive genes are characterized by a decrease in functional activity. At least most of the "biochemical mutants" of *Neurospora* so far obtained are characterized by a loss of synthetic ability and accordingly, by analogy with other organisms, we would expect them to be recessive. Actually, it is possible to test this expectation experimentally. As is the case with many other fungi (Hansen, 1938), hyphae of different genetic strains of *Neurospora* will fuse and form "heterocaryons", i.e., hyphae in which two or more genetic types of nuclei are present (Lindegren, 1942, Dodge, 1942). Physiologically, the heterocaryotic condition is essentially similar to the heterozygous diploid condition in higher forms in which the single nucleus contains two alleles of a given gene. This phenomenon of heterocaryosis in *Neurospora* makes it possible to test mutant genes for what corresponds to dominance in diploid forms. For example, two genetically and morphologically different mutant strains will form heterocaryons which will grow normally if both genes are recessive since each cell will then have, in different nuclei, the normal alleles of both mutant genes. Normal growth forms have been obtained in a number of cases in which such tests have been made. The genes controlling these morphological characters are therefore recessive. Similar heterocaryons have been made with several biochemical mutants. For example, the heterocaryon of the nicotinicless and p-aminobenzoicless strains will grow normally on unsupplemented medium containing neither of the two vitamins. The heterocaryotic mycelium must therefore be capable of synthesizing both vitamins, and both

mutant genes are therefore recessive. Both mutant types can be recovered unchanged from single hyphal tips of such mycelia. This type of evidence indicates that in general the mutant forms of genes concerned with specific syntheses are, as we would expect, recessive.

Since many of these mutant strains of *Neurospora* have been found to be concerned with the synthesis of known chemical substances, often of relatively simple nature, we have at hand particularly favorable material for a biochemical approach to the general problem of how genes act. One method of attacking this problem is based on the general concept of gene action, illustrated by eye-pigment development in Drosophila, that a specific gene controls each step in the series of reactions that constitutes a biosynthesis. The mutation of any one of the genes concerned in such a series will lead to the same end result, modification or failure of the specific synthesis. It follows from this that the mutation of any single gene will break the series of reactions at only one specific place. Accordingly, the problem may be attacked by attempting the isolation of the precursor normally entering into the specific reaction blocked by the gene. Another possibility is to test the activity of all possible intermediates in the synthesis, and in this way to try to establish the normal course of biosynthesis and then to identify the specific step blocked by the mutant gene. This latter approach has been attempted with the p-aminobenzoicless mutant (Tatum and Beadle, 1942). At present we can say only that the synthesis of this vitamin in *Neurospora* probably involves the introduction of the nitrogen into an aliphatic molecule, and that the aminobenzoicless gene is involved either in this step or in the subsequent formation of the aromatic ring.

The last step in the biosynthesis of two vitamins, thiamin and pantothenic acid, is known. In both cases this involves putting together the two halves of the vitamin molecule. The chemical combination of the thiazole and pyrimidine components to give thiamin apparently cannot be performed by the thiaminless mutant of *Neurospora*. Beta-alanine and the substituted butyric acid component of pantothetic acid seem not to be made into this vitamin by the pantothenicless mutant. In these two cases, therefore, the mutant genes appear to control these final steps in the syntheses. The thiazoleless mutant, on the other hand, cannot synthesize the thiazole component, but can put the two halves of the thiamin molecule together.

The amino-acid-deficient mutants, since in each case the synthesis of only one amino acid is deficient, must be concerned with the

synthesis of the characteristic carbon chains or rings rather than with the introduction of the alpha-amino group or the production of the carboxyl group both of which are common to all amino acids. Dr. D. M. Bonner has some evidence indicating that leucic acid can replace leucine with the leucineless mutant. Leucic acid has the branched carbon chain characteristic of leucine, and should easily be converted into leucine through the keto-acid by amination or transamination. This hypothesis seems to be substantiated by the fact that each of the amino acids for which mutants have so far been obtained has a specific group characterized by the presence of organic sulfur, a branched carbon chain, a ring structure, or a nitrogen-containing group in a position other than alpha to the carboxyl group. It seems to be more than a coincidence that all of these amino acids are also essential to the rat, i.e., are needed but cannot be synthesized. Some methionine-less mutants seem to be unable to introduce the methyl group into homocystine, even in the presence of choline, while others which can use either cystine, homocystine, or methionine, cannot introduce sulfur into the molecule.

If each biosynthesis involves, as seems likely, a series of reactions, a number of genes should be concerned with each synthesis, and it should be possible to block a given process in any one of a number of different places. In several instances we have obtained strains of independent origins in which a given synthesis is defective. In some cases these are genetically and physiologically identical, but in others the two mutations are different genetically and grow normally as heterocaryons. Presumably in these the synthesis is blocked at different steps in the two mutants. As an example, we have two genetically and physiologically different mutants in which nicotinic acid synthesis is defective. If all the genes concerned in a given synthesis are mutable we should then be able to estimate the number of steps involved. It should then also be possible to determine the course of the biosynthesis and the type of reaction controlled by each gene by the isolation of intermediate products. These, if diffusible, can be detected by using appropriate strains for specific bioassays.

Theoretically it is important to see whether all physiologically identical genes are also identical genetically, and whether all mutations at the same locus control the same reaction. Allelic genes have been postulated to control the same reaction but to differ quantitatively. *Neurospora* should make it possible to answer some of these questions.

It is becoming more and more firmly established that the funda-

mental nutritional requirements of all organisms are the same for at least many water-soluble growth factors, vitamins, and amino acids. The apparent varying needs of different organisms for these substances actually are only expressions of a need for an outside supply, and finally of an ability or inability to synthesize them from simpler constituents (Peterson, 1941).

Certain lines of evolution, particularly in microorganisms, have been correlated with progressive loss of synthetic ability (Lwoff, 1938, and Knight, 1936). The resulting increased complexity of nutritive requirements is apparently associated with the increased complexity of the environment and consequent increased availability of more and more complex nutritive factors. It would seem that the genetic control of syntheses of growth factors and vitamins which has been demonstrated in *Neurospora* indicates the genetic basis for this general evolutionary loss of synthetic ability.

It is as yet impossible to define exactly the relations of genes to the processes of development and differentiation. The course and orientation of these processes must, however, depend on the activities of a complex of enzyme systems. The syntheses and specificities of these enzymes are presumed to be controlled by the potentialities of the genes concerned, and the time of their production and action is determined by the developmental state already attained. Development and differentiation involve such a high order of organization that experimental analysis is as yet impossible in terms of specific reactions. Each of the reactions into which these complex processes should in time be resolvable, however, is probably fundamentally similar to the relatively simple reactions studied in *Neurospora*. It should therefore be possible eventually to analyze these processes in terms of relatively simple gene-controlled reactions and syntheses.

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BIOCHEMICAL AND PHYSIOLOGICAL ANALYSIS OF ORGANIZER ACTION

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It has been clearly shown in the development of a number of forms that one part of the embryo may exert an influence upon another part so that the latter is induced to differentiate along certain specific lines. In the development of the amphibian eye, for example, the eye cup induces the overlying epidermis, with which it comes in contact, to thicken and ultimately to become lens. It can be demonstrated in a number of ways that this phenomenon is a specific induction. If the eye cup is surgically removed, lens will either fail to develop altogether or will form only imperfectly. Moreover, if tissue normally destined to become something other than lens is brought into contact with the eye cup, the presumptive fate of such tissue is altered and lens can be induced to form. In some species of amphibia, it is true, extirpation of the eye cup does not result in failure of the lens to differentiate, but the inductive influence of such eye cup material can nevertheless be demonstrated if it is allowed to operate on competent ectoderm from another species.

The literature of experimental embryology contains numerous descriptions of such phenomena of dependent differentiation, induction, or organizer action (Needham, 1936; Spemann, 1938; Weiss, 1939; Waddington, 1940; Annual Reviews of Physiology, 1939-1942), but none has been more widely studied nor is any so amenable to the application of biochemical and physiological methods as the so-called primary organizer in amphibian development.

It seems clear in the amphibia, as well as in a number of other vertebrates, that differentiation of the central nervous system and the laying down of the primary axial pattern of the future embryo result from an influence exerted on the presumptive neural plate by the chorda-mesoderm which invaginates at the region of the dorsal blastopore lip during gastrulation. When chorda-mesoderm from the dorsal lip region of an early gastrula is placed in the blastocoele of a second gastrula of about the same age, the transplanted chorda-mesoderm comes to underlie the ventral ectoderm, and the latter tissue, instead of developing into epi-

dermis which is its normal presumptive fate, is induced to differentiate as nervous tissue. The neural axis so formed, together with certain accessory structures, thus develop as a virtual secondary embryo (Spemann and Hilde Mangold, 1924).

It is impossible here to review the abundant experimental evidence for the inductive action of the dorsal lip of the amphibian gastrula, but, in general, there is agreement that presumptive neural ectoderm when deprived of the inductive influence of the chorda-mesoderm, as for example in the transplantation and exogastrulation studies of Holtfreter (1933*a* and *b*), will fail to differentiate as nervous tissue. Such tissue, in the absence of chorda-mesoderm, is apparently incapable of self-differentiating into its presumptive derivative—neural tissue—and remains either purely ectodermal or shows only the general histological characteristics of epithelium. However, when ectoderm of the presumptive neural plate, or even indifferent ectoderm, is acted upon by chorda-mesoderm, neural differentiation will occur.

Following the demonstration in Spemann's laboratory that dorsal lip material from one species of newt would induce neural structures in the ectoderm of another, and that reciprocal effects could even be obtained between the embryonic tissues of urodeles and anurans (Geinitz, 1925), a number of workers showed that cellular debris left after chorda-mesoderm had been crushed or otherwise mutilated, had positive inducing action. This observation indicated that maintenance of intact cellular or tissue architecture seemed to be unnecessary in inductive phenomena, just as it seemed clear from the previous studies that genetic and physiological differences between the inducing and reacting tissues were of secondary importance. These observations were climaxed by the work of Holtfreter (1933*c*) in which he showed that dorsal lip material which had been killed still possessed inducing power.

The suggestion, strongly implicit in the results of these experiments, that induction consisted in the application to the presumptive neural material of some kind of chemical stimulus emanating from the chorda-mesoderm, led naturally to attempts to isolate, by a variety of extraction procedures, and to identify the chemical or chemicals involved. The first success along this line was reported by Spemann, Fischer, and Wehmeier (1933) who obtained positive evidence of induction when an acetone-alcohol extract from the unfertilized egg of Triton was applied in a semi-solid vehicle, such as agar or egg albumen, to the ectoderm of an early gastrula.

Needham, Waddington, and Needham (1934), continuing the approach begun by Spemann, made ether extracts of neurulae which proved to be active. Since various animal tissues had been shown by Holtfreter (1933*d*; 1934) to possess inducing power, mammalian liver was used by the Cambridge group (Waddington, Needham, Nowinski, and Lemberg, 1935) as an abundant source from which an active material could be extracted. These workers were able to trace the activity into the ether-soluble, non-saponifiable, digitonin-precipitable fraction. It seemed, therefore, fairly possible that a sterol, or a substance steroid in character, might be the active inducing agent normally present in living tissue, and this view was made tenable by Waddington and D. M. Needham's (1935) discovery that positive evidence of induction was obtained when synthetic polycyclic hydrocarbons were implanted under the blastocoele roofs of early amphibian gastrulae. More recently Waddington (1938) has obtained excellent, induced neural structures with a series of estrogenic and carcinogenic hydrocarbons.

Another line of attack on the problem emanated from an important observation by Woerdemann (1933*a*) that whereas the glycogen content of the cells in the animal pole region of the blastula was essentially uniform, the cells of the invaginating archenteron roof showed a practically complete disappearance of histochemically demonstrable glycogen, so that a virtual "Glykogen-Grenze" existed at the margin of the dorsal lip. Although meeting with some objection on technical grounds, the observation was confirmed by Heatley (1935) and Heatley and Lindahl (1937) when, with a microtitrimetric method, they showed that glycogen decreased by a significant amount in the dorsal lip region, but in no other, during the transition from blastula to gastrula.

Woerdemann (1933*b*) concluded that induction by the primary organizer was associated with glycolysis, and he was able to demonstrate that tissues with a high glycolytic rate, such as rat carcinoma and Peyton Rous chicken sarcoma, were strongly inductive when implanted into amphibian gastrulae.

Meanwhile positive inductions were obtained with glycogen by Fischer and Wehmeier (1933), and the Freiburg workers were inclined to regard glycogen as the naturally occurring organizer substance. Woerdemann (1933*c*) and Holtfreter (1933*d*), however, were unable to confirm this, and finally Waddington, Needham, Nowinski, and Lemberg (1935, 1936) showed that the activity of crude glycogen

preparations was undoubtedly due to the presence of ether-soluble material adsorbed on them.

At the same time, a number of other workers were investigating the inductive influence of additional chemical compounds. Fischer and Wehmeier, and their co-workers (cf. Spemann, 1938, p. 231 ff.), obtained positive results with fatty acids, nucleoproteins, and muscle adenylic acid, while Barth (1934, 1938, 1939) found cephalin, digitonin, and denatured protein to be effective. Work along this line was climaxed by Okada's (1938) finding that inorganic substances—silicon and calcium carbonate—could induce neural structures.

It had been hoped that the procedure of implanting tissue extractives or pure, synthetic chemicals as substitutes for dorsal lip material would lead ultimately to the identification of the inductor substance or substances normally operative in the reaction between chorda-mesoderm and overlying presumptive neural ectoderm. However, when it was found that neural inductions could be produced by such a wide variety of unrelated chemical substances it soon became apparent that the possibilities for success along this line were distinctly limited. It is true that in some cases, where inductive action has been described, the evidence for induction is not beyond question for the neuroid structures produced appear to be of very poor quality and do not at all approach normal neural tubes in degree of histological differentiation. Yet it seems fairly certain that a number of different chemical substances can stimulate ectoderm to go through at least the incipient stages of neural differentiation. The work reported above does more, therefore, to emphasize the extreme reactivity of embryonic ectoderm than to provide information on the chemical nature of the naturally occurring inductors.

A possible explanation as to why so many varieties of chemical seem to be able to induce neural or neuroid structures in competent non-neural ectoderm stems from an observation by Holtfreter (1933*c*) that tissues which ordinarily do not possess inducing ability can acquire it providing they are treated in certain ways. Thus, ventral ectoderm, which in most of the work reported above served as the test object for inductive effects, can itself induce after it has been killed by boiling, or by alcohol, or, in short, by any method capable of causing protein denaturation.

The Cambridge school (Needham, 1936) used this fact as part of the basis for a theory that an inactive inductor complex, tentatively

identified as consisting of a combination of protein, sterol, and glycogen, exists in all amphibian embryonic tissues, and that this complex is normally broken down so as to liberate the active component in chorda-mesoderm, but in no other region. Even in other parts of the gastrula, however, the active component can be released by a variety of procedures. Thus agents which injure cells or cause cytolysis, as Okada (1938) and Barth (1939) have suggested, can liberate the active agent. Likewise, as shown by Waddington, Needham, and Brachet (1936), the active agent can be unmasked in ventral ectoderm without cytolysis by subjecting the tissue for an appropriate time to the action of methylene blue (see also Beatty, de Jong and Zielinski, 1939).

If the hypothesis of the presence of an inactive organizer complex in all of the regions of the amphibian gastrula is correct, one can never be certain whether a given artificial chemical inductor is working on ectoderm directly, as a morphogenetic hormone, or indirectly by releasing, through cell injury or some other process, the active component of the inactive complex.

On the assumption that information of value relative to the direct or indirect action of various chemicals could be obtained by a consideration of the minimal effective dosages of substances capable of inducing, Shen (1939) made a careful study of the activity of the water-soluble sodium salt of 1, 2, 5, 6-dibenzanthracene-α, β-endosuccinate. He obtained a maximum activity (ca., 50 per cent neural tubes in the experimental series) at a dose slightly in excess of 0.01 μg. per embryo, corresponding to 0.25 mg. per kilo. Even at much lower dosages, however, there was noticeable activity. Shen has calculated (see also Waddington, 1940, p. 30) that most other substances, on the other hand, had to be used in dosages 125 to 250,000 times greater, and even then the effects produced were oftentimes comparatively weak, consisting merely of palisadings of cells.

Recently, Shen (1941) studied the effect of cultivating ectodermal explants in salt solution to which the endosuccinate derivative of 1, 2, 5, 6-dibenzanthracene had been added. In more than 60 per cent of the explants so treated, neural structures developed in the complete absence of any solid implant so that the possibility of induction by mechanical means was completely excluded, and the adequacy, as in inductor, of chemical action alone was demonstrated.

Waddington (1940) concluded that the steroid substances are the most potent artificial inductors yet found; that they probably are

closely related to the naturally occurring morphogenetic hormones present in chorda-mesoderm, and that their action, in contrast to that of such substances as digitonin, nucleoprotein, fatty acids, or cephalin, is direct.

An alternative explanation of the fact that such a wide variety of chemical agents can be found which will induce neural structures in indifferent ectoderm is that neural tube formation is a general response by competent tissue to non-specific activation (Child, 1941). Induction, according to this view, can be accomplished by any one of a number of stimulating agents which start a series of processes leading, as an end-result, to neural tube formation.

Although there is evidence to support such a view, it would seem that induction involves something more than localized, non-specific stimulation. It will be recalled that Spemann (1938) demonstrated regional differences in the inductive ability of the chorda-mesoderm and concluded that the details of the differentiation process depended as well upon the character of the inductor, i.e., head-, trunk-, or tail-organizer, as upon the nature of the reacting tissue. Somewhat similar evidence was obtained by Eakin (1939) in a study of regional characteristics in the organizer of the trout, and regional differences are likewise indicated in the work of Lehman (1937) and Hall (1942). A certain degree of specificity in the inductive action of adult mammalian and amphibian tissues on isolated gastrula ectoderm of *Triton alpestris* has been reported by Chuang (1939).

In this connection it is of interest to note that even with artificial inductors, different degrees of response can be obtained. Shen, in the work reported above, has shown that with lower dosages of "endo-succinate," simple palisadings of cells are produced, but that the tissue responds to somewhat stronger dosages by the formation of neural tubes.

The question of the type of stimulus necessary for neural tube formation has again been raised by the results of recent work by Barth (1941) in which he has described neural differentiation without organizer action. Barth found that large ectodermal explants from amphibian gastrulae would form neural structures in a high percentage of cases when the anterior-posterior polarity relations of the explant were maintained. In this work, the neural tubes were formed from the anterior end of the explant, i.e., the end located at or near the animal pole in the intact gastrula. However, when the polarity of the explants

was experimentally obliterated, or when the size of the explant was reduced, the frequency of neural tube formation was considerably lessened. Barth has interpreted these findings as suggesting the existence of a gradient in neural differentiation extending from the animal to the vegetal pole in the embryo. It would appear, however, that the maintenance of polarity in the explants is of only secondary importance in neural differentiation in view of the high frequency (4-29%) of neural tubes obtained when polarity was experimentally abolished.

It is noteworthy in the above work that many, although not all, of the explants were taken from very late gastrulae in which it may be considered that invagination is almost complete (Harrison's stages 11-13). The more anterior ectoderm from such gastrulae would, at least in part, represent presumptive neural material and it would appear highly probable that such ectoderm might be contaminated by mesodermal cells, or might already have been acted upon inductively by the chorda-mesoderm. It is of interest in this connection to recall the results of experiments by Emerson (1942) where late gastrula ectoderm (which had already been underlain by mesoderm) was explanted to the site of regenerating tissue in *Rana pipiens* larvae. The differentiation of neural structures (atypical brains, lensless eyes, etc.) was confined to the presumptive neural plate material, while presumptive epidermis formed only definitive epidermis or epidermal derivatives. It thus appears that presumptive neural plate, once it has been acted upon by the inductive influence of the chorda-mesoderm, is able to complete its differentiation into neural tissue even though the inductive influence be discontinued.

Side by side with the biochemical assay of the various regions of the gastrula, and the study of the reaction of competent ectoderm to the stimulating action of substances whose chemical composition is known, it has been felt that valuable information relating to induction phenomena could be gained by a consideration of the metabolic characteristics of various regions of the gastrula. Thus a comparative study of the metabolic processes in the dorsal lip—or organizer region—and in indifferent ectoderm might serve to increase our knowledge of how the chemical substances believed to be active as inductors are synthesized and released.

Initial information along this line was provided by Brachet (1934*b*), in a semi-quantitative study, when he found that destruction by microcautery of the dorsal lip region in *Rana temporaria* gastrulae resulted

in a greater diminution of oxygen consumption than when a similar area of ventral ectoderm was destroyed. A comparison of the rate of carbon dioxide production of isolated pieces of dorsal lip and ventral ectoderm gave similar results, the carbon dioxide output by the dorsal lip being some 80 per cent greater than that of the ventral ectoderm.

In later work Brachet (1936) made an estimation of the size of the isolates from *Discoglossus* gastrulae in terms of their total nitrogen content, and the results again indicated a more abundant carbon dioxide production by the dorsal lip region than by ventral ectoderm.

At the same time, Waddington, Needham, and Brachet (1936) measured the oxygen consumption of explants of dorsal lip and ventral ectoderm, relating the figures obtained to the dry weights of the tissues. In this study, the rate of oxygen consumption per unit weight of the two regions proved to be essentially similar.

Brachet and Shapiro (1937) measured simultaneously the rate of oxygen consumption of the two halves of intact gastrulae of *Rana sylvatica* and found a 47 per cent difference in favor of the half containing the dorsal lip.

In two series of experiments on *Amblystoma mexicanum,* the oxygen uptake of the dorsal lip region was found, by Fisher and Hartwig (1938), to be 28 per cent and 11 per cent greater than that of ventral ectoderm.

Using the Cartesian diver technique, Boell and Needham (1939) found the average oxygen consumption of dorsal lip and ventral ectoderm from *Discoglossus pictus* and *Amblystoma mexicanum* gastrulae to be approximately the same, while a 26 per cent difference in favor of the dorsal lip was reported by Boell, Koch, and Needham (1939) in a second series of determinations on *Amblystoma mexicanum.*

In his latest work, Brachet (1939) reported that isolates of dorsal lip and ventral ectoderm from *Rana fusca* gastrulae respire at essentially identical rates, but that in *Discoglossus* the oxygen consumption by the dorsal lip is some 30 per cent in excess of that of the ventral ectoderm. Finally, Needham, Rogers, and Shen (1939) report identical average values for the respiration of dorsal lip and ventral ectoderm from *Rana temporaria* gastrulae.

The experiments reported above, and summarized in Table 1, were performed on embryonic material from different species, and the conditions of the experiments—as to temperature, duration, and physio-

TABLE 1

SUMMARY OF WORK ON RESPIRATORY RATES OF DORSAL LIP AND VENTRAL ECTODERM IN AMPHIBIAN GASTRULA

Investigator	Material	Rate DL	Rate VE	DL/VE
Waddington, Needham and Brachet	*T. alpestris*[3]	0.23	0.21	1.10
Brachet and Shapiro	*R. sylvatica*[2]	85	58	1.47
Fischer and Hartwig	*A. mexicanum*[3]	2.34	1.83	1.28
Fischer and Hartwig	*A. mexicanum*[3]	2.13	1.92	1.11
Boell and Needham	*Discoglossus*[4]	4.80	4.93	0.98
Boell and Needham	*A. mexicanum*[4]	3.21	3.18	1.01
Boell, Koch and Needham	*A. mexicanum*[4]	5.3	4.2	1.26
Brachet	*R. fusca*[5]	0.164	0.153	1.07
Brachet	*Discoglossus*[4]	4.1	3.1	1.31
Needham, Rogers and Shen	*R. temporaria*[4]	3.74	3.78	0.99

Respiratory rates (μl. O_2 consumed per hour) are based on the following units of tissue: (1) per milligram dry weight; (2) per gram wet weight; (3) per 10 milligrams dry weight; (4) per milligram nitrogen; (5) not stated by the author.

logical medium—were not identical, but it is unlikely that these factors were solely responsible for the lack of agreement in the results.

Brachet (1939) concluded that the differences in the rates of oxygen consumption of dorsal lip and ventral ectoderm are much less marked in experiments of short duration than in longer experiments. Yet it would seem that the dissimilarities in the results shown in Table 1 are almost certainly due to a more fundamental difference in technique arising from the failure of the various workers to use corresponding parts of the gastrula for comparison. So-called dorsal lip material varies in composition with the advance of the gastrulation process. In the very young gastrula, the region of the dorsal lip consists exclusively of chorda-mesoderm which has yet to be invaginated; in the later gastrula, it is composed of a double layer of tissue, the outer one representing presumptive neural plate and the inner layer comprising chorda-mesoderm. The term "ventral ectoderm", moreover, denotes no clearly definable zone in the gastrula, and it is highly probable that a piece of ventral ectoderm taken from near the animal pole will show a rate of respiration different from that of tissue removed from nearer the opposite pole.

Hence, it is to be expected that the nature of the results obtained would depend upon the particular region of the ventral ectoderm selected for comparison with the dorsal lip, as well as upon the age of the gastrula from which the pieces were taken, and the reproducibility of a given result would depend upon the precision with which corre-

sponding regions could be isolated for study from different gastrulae by different workers.

In order to circumvent some of these difficulties, the entire cortex of the gastrula was dissected into regions consisting of various presumptive areas, as shown in Figure 1, and the oxygen consumption of the

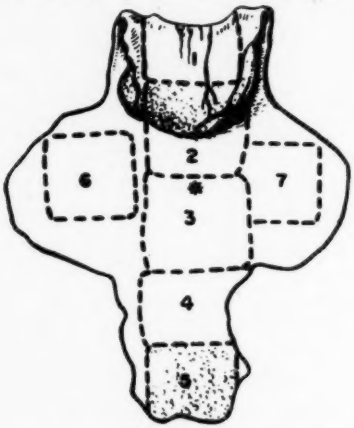

FIGURE 1

DIAGRAM SHOWING METHOD OF DISSECTING THE GASTRULA OF *Amblystoma punctatum*

In the dissection, the inner yolk-mass is removed, and the cortical part of the gastrula divided into seven or more zones as indicated. The asterisk denotes the animal pole of the gastrula and is marked, prior to each operation, by puncturing the tissue in this region. Each of the pieces so isolated is transferred to the diver bulb with 1.5 µl. Holtfreter solution.

pieces was then measured in the Cartesian diver apparatus by the procedure described by Boell and Needham (1939).[1]

The results of a typical experiment are shown graphically in Figure 2, and indicate without question that differences of considerable magnitude appear in the oxygen consumption of different areas from the same gastrula. There seems to be a gradient in respiratory activity extending from animal pole to vegetal pole with the more anterior part of the presumptive neural plate and the anterior ectoderm showing the highest rate of oxygen consumption. As might be expected, some differences in the absolute levels of the respiratory rates of the various

[1]A preliminary report of the experiments on which this section is based was made by Boell and Nicholas (1940). A more complete presentation of these and additional data will shortly appear.

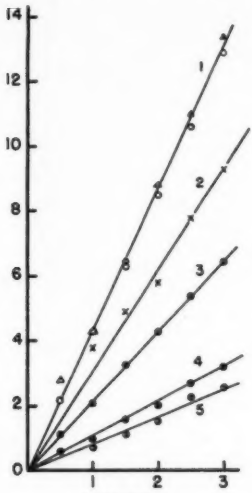

FIGURE 2

RESULTS OF A TYPICAL EXPERIMENT SHOWING OXYGEN CONSUMPTION OF DIFFERENT PRESUMPTIVE REGIONS OF AN EARLY GASTRULA (¼-moon yolk-plug stage)

Curve 1=anterior ectoderm (Δ), and the anterior part of the presumptive neural plate (O); Curve 2=posterior ventral ectoderm; Curve 3=dorsal lip region (chorda-mesoderm); Curve 4=yolk endoderm; Curve 5=anterior portion of invaginated chorda-mesoderm. Ordinate denotes oxygen consumption of the explants in mμl. per μg. nitrogen; abscissa shows time in hours.

regions are apparent from gastrula to gastrula, but the relative differences among the regions are, in general, similar to those shown in Figure 3.

Since dorsal lip material represents a double layer of tissue while the other regions selected for study are generally thinner, it might be questioned whether sufficient oxygen from the air in the gas phase of the divers was diffusing into the thicker tissue to maintain respiration at its potential maximum. In order to obtain information on this point, the oxygen uptake of gastrula explants was measured in air and in pure oxygen. The results of these experiments show clearly that the differences indicated above are due to intrinsic dissimilarities in the tissue rather than to differences in the availability of oxygen.

It is well known that a gradient in the distribution of yolk exists in the amphibian egg (Bragg, 1939; Daniel and Yarwood, 1939) and it would not appear unlikely, therefore, that the differences in respiration

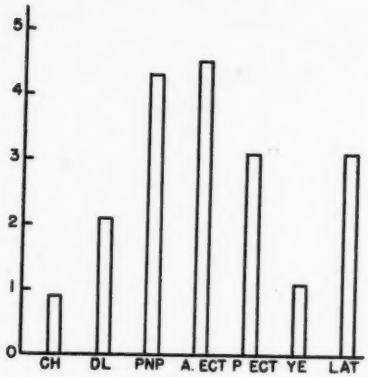

FIGURE 3

RESPIRATORY RATES OF VARIOUS REGIONS OF THE GASTRULA FROM EXPERIMENT SHOWN IN FIGURE 2

Ordinate represents Q'_{O_2} ($m\mu l$ O_2 per μg. nitrogen per hour). The symbols on the abscissa denote respectively chorda-mesoderm, dorsal lip region, presumptive neural plate, anterior ectoderm, posterior ectoderm, and lateral ectoderm.

reported above might simply reflect the presence of varying amounts of yolk in the parts of the gastrula selected for study. In order to obtain information on this point, a quantitative study of the yolk content of the various regions has been made. When pieces of gastrula are centrifuged at high speeds in the Beams air-turbine centrifuge, a complete disruption and stratification of the tissue occurs, and the amount of yolk can be estimated volumetrically. When the volume of yolk is subtracted from the initial volume of the tissue, a fairly accurate approximation of what might be called yolk-free or active substance can be gained, and respiration can then be calculated in terms of such "active material." In general, as shown in Table 2, there is an inverse relationship between the yolk content of the various regions studied and the rate of respiration, i.e., respiratory rate varies directly with the amount of "active material." As can be seen from the table, when respiration is calculated on the basis of "active material" ($Q^{ac}_{O_2}$), the differences in the rate of oxygen consumption among the various regions, though not abolished, are considerably reduced. Results which are in essential agreement with those reported above have recently been presented by Barth (1942).

It is of course obvious that introducing a correction for the yolk con-

TABLE 2

RESPIRATION OF VARIOUS REGIONS OF THE GASTRULA WHEN CORRECTED FOR YOLK CONTENT

Region	No. Expts.	Q'_{O_2}	%*	No. Det.	% Yolk	% Active	$Q^{ac}_{O_2}$	%*
Dorsal lip	31	2.1	43	7	56	44	4.8	66
P. neuralplate	14	4.9	100	7	33	67	7.3	100
Ant. ectoderm	20	4.5	92	7	31	69	6.5	89
Post. ectoderm	12	2.8	57	7	43	57	4.9	67
Yolk endoderm	13	1.3	27	8	66	34	3.8	52
R. Lat. ectoderm	3	3.1	63	2	34	66	4.7	66
L. Lat. ectoderm	6	3.4	69	2	34	66	5.1	70
Chorda:	6	1.2	25	2	63	37	3.3	45
anterior		1.1						
middle		1.1						
posterior		1.9						

*The respiratory rates have been expressed as percentages of the rate for presumptive neural plate.

tent of a given piece of tissue gives no more than an approximation of the amount of active material in the tissue. Certainly, it is likely that still a part of the so-called "active material" is not primarily important in a respiratory sense. If it were experimentally possible to determine accurately the oxidatively active protoplasm in the various regions and then to calculate the rate of respiration on this basis, it is possible, but not certain, that the differences in respiration would become even less marked.

The question naturally arises as to whether such quantitative differences in metabolism are significant from the standpoint of induction phenomena. Child (1941) has recently made the suggestion that "in general, the natural inductors are, or at certain developmental stages become, high gradient levels." According to this view, "the inductor apparently represents a higher range of gradient-levels and by raising the level of presumptive neural plate, makes possible further development." However, the data do not seem to support such an interpretation, for the primary inductor——chorda-mesoderm——far from having the highest rate of respiration in the developing system, actually is seen to have a low rate, while the presumptive neural plate, instead of representing a lower level, shows a higher respiratory activity than any other part of the gastrula.

Furthermore, it would seem unlikely that a quantitative level of metabolism, or difference in level, is necessary in induction phenomena since Brachet (1939) showed that induction could occur in the presence of cyanide and under anaerobiosis. Moreover, Philips (1942)

found no differences of significance in the rate of oxygen consumption of the various regions of the early chick blastoderm, and Lindahl and Holter (1940) found that no quantitative differences were demonstrable in the respiratory rates of animal and vegetal halves of sea urchin embryos (*Paracentrotus lividus*) in spite of their different developmental potentialities.

We are not yet in a position to reach a definite conclusion as to the importance of quantitative levels of respiratory activity in induction, but it seems not unlikely that the quantitative differences in respiration in the regions of the amphibian gastrula are either largely fortuitous or of only secondary importance.

The results obtained by Woerdemann (1933*a*), Heatley (1935), and Heatley and Lindahl (1937), to the effect that glycogen disappears locally in the dorsal lip region during gastrulation, makes it highly probable that organizer action is in some way linked with carbohydrate metabolism. Consequently, a study of the qualitative aspects of metabolism in the various regions of the gastrula would be of considerable interest. Such a study would have additional significance in view of the hypothecated existence of an inactive organizer complex consisting of protein, sterol, and glycogen.

Using the Cartesian diver apparatus, Boell, Koch, and Needham (1939) found that blastula pieces had a respiratory quotient of approximately 0.75, a result in harmony with Brachet's (1934*a*) report of respiratory quotients of about 0.7 for intact blastulae. As soon as gastrulation had commenced, the respiratory quotient of the dorsal lip region rose to unity, and this high value was maintained through at least the closing neural fold stage. The respiratory quotient of ventral ectoderm, on the other hand, although showing some tendency to rise during gastrulation, averaged approximately 0.8. Confirmation of this result by Brachet (1939) and Needham, Rogers, and Shen (1939) leaves little doubt that the respiratory quotient of dorsal lip material is approximately unity while the value for ventral ectoderm averages about 0.8.

A further indication of the existence of qualitative metabolic differences in the various regions of the gastrula is seen in data on the rate of anaerobic glycolysis (Boell, Needham, and Rogers, 1939). Dorsal lip material showed a $Q_L^{N_2}$ value of 0.63 while the value for ventral ectoderm was only 0.21, and statistical analysis revealed that this difference was highly significant.

That differences in protein metabolism may likewise exist in various parts of the gastrula is suggested by the fact that the anaerobic ammonia production is three times greater in the dorsal lip than in ventral ectoderm (Boell, Needham, and Rogers, 1939) and by Brachet's (1938) demonstration of local accumulation of sulphydryl groups in the organizer region.

Conclusions

We do not know what the chemical entities involved in embryonic induction actually are, although it has been suggested that steroid substances may be responsible. In view of the fact that ventral ectoderm contains natural inductors in bound or conjugated form, it is not surprising that many different chemicals have been found to have inductive action. It is believed that such artificial inductors, in many cases, work indirectly by releasing the active principle from the organizer complex. It seems clear, therefore, that it is futile to bring more and more chemical substances into contact with embryonic ectoderm and to select, from the galaxy of those that work, the one or several which may have a counterpart in the inducing tissue of the embryo. A more profitable approach to discovering the chemical identity of naturally occurring inductors would seem to be the preparation and testing of active and inactive fractions from embryonic tissue.

It is at present impossible to indicate the precise relationship between metabolism and induction. Quantitative differences in metabolism, although present in the various parts of the gastrula, appear to be of secondary importance. Of greater interest, perhaps, are the differences in the kind of metabolism which exist between inductor and indifferent regions of the embryo. It would seem hardly without significance that release from the chorda-mesoderm of the substance or substances concerned normally with neural induction in amphibian development occurs in a region of the gastrula characterized by a predominant metabolism of carbohydrate.

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DEVELOPMENTAL RESPONSES IN ISOLATED PLANT TISSUE SYSTEMS

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The subject of this year's symposium is growth as a phenomenon of response to formative agents, both chemical and physical. I have been assigned the task of introducing this subject in so far as higher plants are concerned. It is intended that my emphasis should be on "normal" growth, while Dr. Riker is to consider pathological growth. I know, however, that I will make occasional forays into his territory, and doubtless he will into mine.

Developmental response becomes evident in any system through the

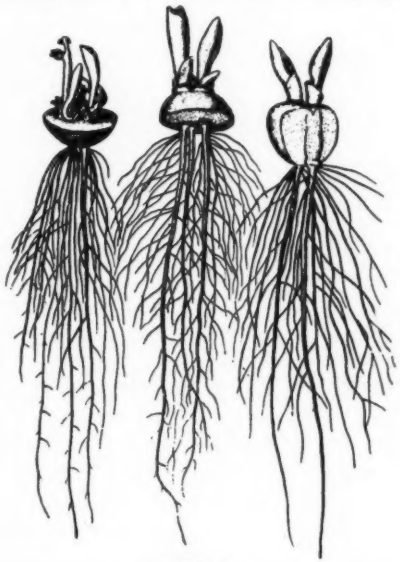

FIGURE 1

Tubers of *Corydalis solida* cut and allowed to regenerate, showing the differences in response of a single tissue according to the spatial relation of the exposed surface to the subjacent tissue mass. The importance of tissue and cell polarity is here shown in a striking manner. (From GOEBEL, K. 1908. Einleitung in die experimentelle Morphologie der Pflanzen, 220, Fig. 111.)

appearance of polarity, asymmetry, pattern. All living creatures evince pattern to a greater or less degree in all their parts. Where no pattern, polarity, or asymmetry is evident, we must conclude either that there are no differentially oriented stimuli acting upon the system—a state which must certainly be very rare indeed—or else that the system is dead. Thus, in any analysis of developmental response we seek to eliminate one by one the differential stimuli until we obtain as nearly as possible an indifferent or at least completely regular response, and then to add known and controllable stimuli, observing the changes in response which result. It is for this reason that the isolation and maintenance of uniform, undifferentiated, patternless masses of tissue as bases for developmental studies take on such importance.

A plant's development progresses as a continually expanding process from the single-celled egg up to the complexly ramified and variously differentiated mature stage. We may approach the problem of morphogenesis at any given level in this process and at each level we will find a complex interplay of stimuli and response. This interplay begins even before fertilization of the egg in an asymmetrical distribution of the egg cell's constituents and in the differential stimuli resulting from its position with respect to the embryo-sac wall, to gravity, etc. The act of fertilization is a unidirectional, polarized stimulus and therefore immediately as well as potentially morphogenetic in character. It is seldom that we can detect an obvious polar response at these early stages, even though we know quite well that such must occur. But, with the first division of the fertilized egg, it is possible to observe the inception of a visible polarity. Even here the task of analyzing the factors responsible for this polarity is extremely complex. Attempts to analyze the early morphogenesis of much simpler, free-swimming egg cells such as those of the brown alga, *Fucus,* present far fewer problems; yet they have led to the implication of pressure, light, gravity, oxidation-reduction gradients, pH gradients, electrostatic gradients, and ultimately though not primarily gradients in auxin concentration, with varying degrees of certainty and clarity. I cite this multiplicity of known or suspected factors merely to show the complexity of the problem even in dealing with simple systems, in order that you may not be too greatly taken aback by our almost complete lack of real knowledge as regards the fundamental bases of morphogenesis in the more complex systems which we encounter in more advanced points in the ontogenetic scale.

With the second pair of divisions there are segregated, in the rela-

tively simple vascular Cryptogams, the mosses and ferns, four cells which give rise to the four primary organs of the embryo: radicle, plumule, cotyledon, and suspensor. In the flowering plants, this segregation is frequently delayed until after a series of transverse divisions have formed a long columnar suspensor. It may sometimes be no longer recognizable at all. One may attribute this delay in segregation to a genetically controlled retardation of developmental stages—a retardation which finds perhaps its most striking botanical expression in the gradual ontogenetic transposition of the meiotic division from the first post-fertilization division in the primitive green algae all the way to the last pre-fertilization division in the highly evolved flowering plants. Or one may attribute it to the obvious increase in the complexity of the surrounding organs and cellular environment. At present we cannot decide between even these simple alternatives. After these first divisions, the mitoses appear for a long time to be oriented largely by mechanical tensions. In the typical dicotyledon, for example, the formation of a slightly flattened, heart-shaped embryo, the elongation of the three arms of this body to form the radicle and two cotyledons, the bending of the whole in conformity with the outlines of the surrounding seed coats and nutritive tissues all require the postulation of no very complex interplay of stimuli to provide fairly plausible explanations. Nor do the first steps in germination indicate any very complex picture from a morphological point of view.

With the inception of the seedling, pre-adult and adult stages, however, there do begin to enter in so many obvious morphogenetic reactions to external stimuli as well as to internal directional forces, in the form of tropistic responses on the one hand and genetically determined developmental patterns on the other, that we begin again to find bases for study. The whole field of growth substance investigation stems from an analysis of these post-embryonic behavior patterns. As regards tropistic responses to external stimuli, the causal sequences are fairly obvious though none the less difficult to analyze in detail. As regards genetically determined patterns, the relations are more obscure, but some are indeed coming to light. Such are, for example, the dependence of dwarf habit in certain species of corn on enzymatic destruction of auxins and the similar dependence of recumbent habit in otherwise erect forms on a similar disturbance of auxin metabolism. Still more complex, but in some respects more interesting, is the dependence of differences in normal branching habit of related species of certain composites on genetically determined differ-

ences in auxin production and distribution. The hormonal control of flowering in certain biennial plants such as burdock (*Xanthium*) places this process likewise in the category of morphoses whose controlling mechanisms are in their broad outlines known and hence apt subjects for further investigation.

So much for the general field, about which I personally know no more than any of you could, if you wished, learn from the current literature.

As I said in the beginning, however, this matter of developmental response is largely expressed in the form of patterns and asymmetries of one sort or another. One of the basic steps in the analysis of such problems is therefore to reduce that asymmetry to a minimum compatible with the continued viability of the material under investigation.

Such a minimum of asymmetry is provided for us naturally in the undivided egg cell and young embryo. It is also provided to a still greater extent in certain tissues such as large endosperms, the piths of some plants, massive fleshy fruits, etc. In theory, if one could experiment with these massive tissues, they should, in this one respect, prove ideal materials for morphogenetic studies. Actually they are not particularly suitable materials for such work, as Haberlandt and his students found to their cost, for they are all tissues which are normally relatively inert, rarely making any proliferative growth. Any morphogenetic expression which we might conceivably evoke from them would be of necessity an abnormal expression. These we know quite well to be possible under certain abnormal circumstances, as Haberlandt himself showed and as Bloch and others have verified, but they represent an approach which presents relatively few aspects that are adapted to experimental investigation. We have long recognized the need for other, more flexible materials. The search for such materials has led in the last third of a century to the development of the so-called tissue-culture technique.

This technique was first outlined and attempted 40 years ago by Haberlandt, using plant materials. Five years later it was first successfully applied, this time to animal tissues, by Harrison when he reduced the neuroblast of the frog to a simple yet viable system in which response could be continuously observed for considerable but not unlimited periods. Carrel perfected the method by developing an environment in which fibroblasts and epithelium could be maintained in a still simpler, almost patternless state for indefinite periods. Carrel and his school emphasized this patternless state so constantly, as an

end rather than as a basis for experimentation, that the idea that these tissues are capable of developmental response has been largely forgotten and even frequently denied. It is only the more recent work of such investigators as Fell, Weiss, and a few others that has brought us back to a realization that here is, in fact, a material ideally adapted to the study of developmental response and morphogenesis.

Thirty years of animal tissue cultures have, it must be confessed, given us far fewer real results in the field of morphogenesis than we might reasonably have expected had investigation not been so consistently turned in a counter direction by Carrel's leadership. What then can we expect of plant tissue cultures which are, in a strict sense, only some three years old? (For one must remember that, although sporadic but unsuccessful attempts are recorded in the literature as early as 1902, it was only in 1934 that the capacity of plant roots to grow in culture for potentially unlimited periods, subject to experimental control, was clearly demonstrated, and it was not until 1939 that Gautheret in Paris, Nobécourt in Grenoble, and I in this country carried out the first successful cultures of undifferentiated tissue masses for like periods.) Three years (or even eight years) are not a long time in which to obtain significant results in a new and complex field. The materials have, however, shown themselves to be potentially valuable for just such morphogenetic problems as those in which we are interested.

Plant	Dominance	Subcultures from branches	Callus	Stem regeneration	Habit	Subcultures
Clover	*Pronounced*	*Difficult*	*None*	*None*		
Tomato	*Moderate*	*Moderately successful*	*Distal only*	*None*		
Radish Dandelion	*Lacking*	*Very easy*	*Both proximal and distal*	*Frequent*		

FIGURE 2

CHART SHOWING THE RELATIONS BETWEEN GROWTH HABIT, DEGREE OF APICAL DOMINANCE, AND NATURE OF REGENERATIVE RESPONSE OF EXCISED ROOTS OF FOUR SPECIES OF PLANTS

First, root cultures. These show, according to the species studied, remarkable differences in degree of polarity and in that type of correlation which we call "dominance." Roots of radish and of most composites show almost no apical dominance, that is to say, the manner and rate of development of lateral growing points is unaffected by the manner and rate of development of the primary tip. The result is a completely haphazard growth, primary and secondary members being indistinguishable from one another. All are equally suitable as material for starting subcultures. Legume roots, such as clover, on the other hand, possess a high degree of apical dominance, the development of laterals being very strongly retarded by the presence of a primary tip, with the result that lateral roots attached to the primary remain short, and upon subculturing show a marked and prolonged inhibition of growth as compared to cultures from primary apices, escaping only gradually from this suppression. Tomato roots are intermediate in behavior between these two extremes. The degree of polarity seems to be in some way correlated with this matter of dominance, for legume roots along with their strong dominance show a high degree of polarity, forming little or no callus at either distal or proximal cut surfaces and never regenerating growing points; tomato roots form excellent callus at distal cuts but not at proximal ones; while roots of composites with weak dominance have an equally weak polarity, forming excellent calluses at both proximal and distal cut surfaces and frequently regenerating stem growing points and leaves. If one could analyze, in these experimentally controllable systems, the factors responsible for these differences in dominance and polarity, one would have made a great step forward toward an understanding of morphogenetic patterns. Such analysis has, so far as I know, not even been attempted.

These are cases of genetically controlled morphoses which can be manipulated somewhat, perhaps suppressed but never entirely eliminated. Roots are, however, in spite of their rather highly developed inherent patterns, extremely sensitive material for the study of many types of experimental morphoses. Most obvious among these are their primary reactions to nutrient variables, both qualitative and quantitative. The basic synthetic nutrient now generally used for plant organs and tissues includes 11 inorganic salts containing 16 elements, carbohydrate, three vitamins (thiamin, pyridoxine, and nicotinic acid), and one amino acid (glycine). All of these elements, with the exception of sodium and chlorine, are known to be beneficial to plant

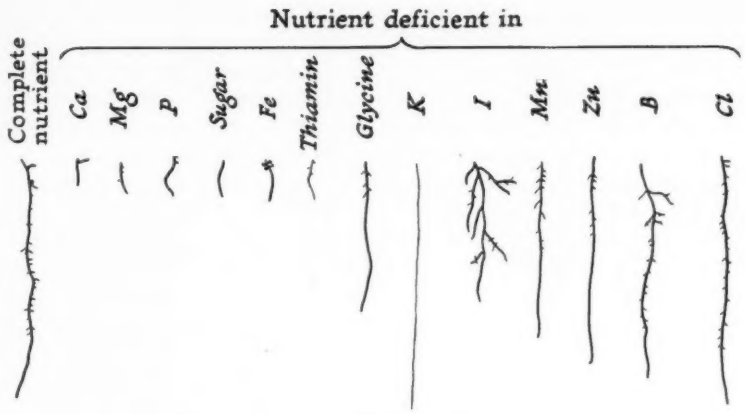

FIGURE 3

SEMI-DIAGRAMMATIC REPRESENTATION OF THE RESPONSES OF EXCISED TOMATO ROOTS TO THE OMISSION OF EACH OF 13 INDIVIDUAL NUTRIENT CONSTITUENTS FROM AN OTHERWISE COMPLETE NUTRIENT

tissue growth. Most of them are known to be not only beneficial to but essential for development, so that their omission results in complete cessation of growth or profound changes in developmental behavior. Thus, omission of calcium, magnesium, phosphorus, or sugar results in abrupt and complete failure; omission of iron results either in complete and immediate failure or at best in the formation of a mass of short, stubby branches; omission of thiamin results in a slowly increasing tendency to sickly, thin, unhealthy growth habit; omission of glycine results in a greatly reduced but not markedly abnormal growth; omission of potassium results in the disappearance of all branching; omission of iodine results in a profusion of stubby, short-lived, repeatedly ramified branches, etc. These are, of course, simple "all or none" studies. The effects may be quantitative as well as qualitative. This is shown in an especially striking manner in the reaction to various concentrations of iron. It is clear from these preliminary observations that such excised roots are capable of serving as excellent experimental material with which to attempt to unravel those morphoses which result from nutritional differences. I think it is perhaps worth noting that almost nothing has been done to attempt to determine the formative effects of even these obviously essential elements on isolated animal tissues.

Since so much of the discussion of plant morphoses has in recent years been tied up with the question of auxin metabolism, I cannot refrain from bringing in some stray bits of evidence on that score. Excised roots certainly grow quite normally without external sources of auxin. There is some evidence both in favor of and opposed to the idea that they may synthesize their own auxin in adequate amount. If they do, it must certainly be in extremely minute amounts. If to a synthetic nutrient capable of supporting normal growth of excised tomato roots, we add various amounts of an auxin (indole acetic acid), we obtain a regular sigmoid curve of response. A concentration of 10^{-11} (one part in ten thousand million parts of water) definitely inhibits growth. Concentrations of 10^{-12} and 10^{-13} may possibly stimulate over-all growth slightly. (The observed difference of 10 to 15 per cent is within the experimental error of the method, so that the apparent stimulation may or may not be significant). Concentrations of 10^{-14} or less are without effect. The quantitative data thus lend no clear support to the idea that auxin may stimulate growth of excised tomato roots at concentrations above those which may be synthesized in the excised tissues themselves. Qualitatively the injurious effect of this substance even in minute traces is likewise clear. One part of indole-acetic acid in two million parts of nutrient results in a marked shortening and thickening of the root axis, the branch roots being crowded together to simulate a rooting response. The cells of the epidermal and cortical layers are swollen, frequently ruptured and necrotic. The *sensitivity* of excised tomato roots to the auxin is thus of the same order as that to the vitamins such as thiamin and pyridoxine, but the reaction is mostly, if not entirely, a reaction typical of that to poisonous materials. This is not to say that beneficial effects may not occur under some conditions. I can only say that I have not found them under conditions otherwise capable of supporting normal growth.

But roots are already highly differentiated structures with patterns, symmetries, polarities which we have not as yet been able to eliminate or even modify to any very great extent. In 1937 I turned my attention to another type of culture. Gautheret and Nobécourt had already had some success in culturing cambial tissues of various sorts. Their cultures, however, grew slowly and were of uncertain significance. The materials they had chosen to work with were cambium from poplar, willow, maple, beech, elm, and pine trees and fleshy stems and roots from carrot, potato, and kohl-rabi. All were normally

slow-growing and relatively stable materials. My own experience has been that materials of these types give cultures which develop slowly, tend to differentiate, and retain a considerable degree of normal morphogenetic pattern under all the cultural conditions to which we have so far subjected them. Gautheret and Nobécourt have likewise reported a considerable persistence of polarity, and even a tendency to bud formation in some cambial cultures, especially those of elm. It seemed probable that materials which had already escaped somewhat from the normal morphogenetic bonds might prove more satisfactory for preliminary studies, and one must remember that all of these *are* preliminary.

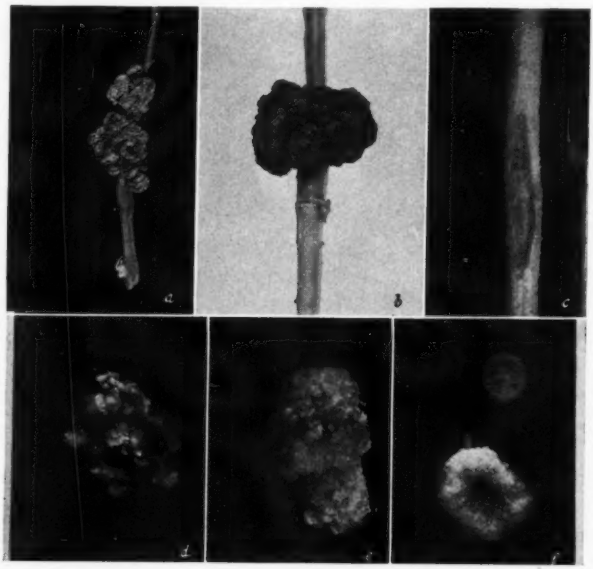

FIGURE 4

"a"—spontaneous, genetically induced gall on a plant of the hybrid, *Nicotiana langsdorffii* x *N. glauca*. (*Pl. Physiol.*, 1942, **17**, 162.) *"b"*—tumor produced on sunflower by inoculation with *Phytomonas tumefaciens*. *"c"*—healing of a wound resulting from implantation of a block of sunflower pith taken from a healthy plant and grafted into a second plant of the same species. No tumor results. Below are shown tissue cultures taken from *"d"*—a genetic tumor on the *Nicotiana* hybrid shown above. X 2. (*Bull. Torrey Bot. Club*, 1939, **66**, 509.) *"e"*—a bacteria-free metastatic crown-gall tumor developed on sunflower as a result of infection elsewhere in the host by *P. tumefaciens* (X 1.1), and *"f"*—healthy sunflower tissue taken from an uninfected plant similar to that shown in the top row. X 1.1. (*Cancer Res.*, 1942, **2**, 602.)

Such materials presented themselves first in the rapid-growing, disorganized galls which regularly appear spontaneously on plants of the hybrid, *Nicotiana langsdorffii* x *N. glauca*. These galls appear first as scattered overgrowths wherever minor wounds occur, such as those resulting from the abscission of a leaf or flower stalk. They can regularly be evoked on leaf or stem by sterile needle puncture, pinching, or even drastic bending. Once started, the overgrowths themselves set up tissue tensions which result in new calluses; these spread, the entire plant ultimately becomes involved and finally dies from sheer mechanical disorganization. This behavior remains unchanged when portions of the hybrid are used either as stock or scion in grafts with plants of either of the parent species and is not transmitted in any degree to the grafted pieces, showing that it is not infectious. The overgrowths appear to result from some genetic disturbance, some chromosomal incompatibility between the two parents. Since their origin lies in the genetic constitution of the plant, all tissues are capable of giving rise to them, so that sources of material for experimentation are theoretically not anatomically limited. Actually the procambial tissues just back of the growing point have proved most suitable for manipulation.

When fragments of tissue a millimeter or more in diameter are removed from the procambial regions of such plants and placed on nutrient agar or other semi-solid nutrient substratum, they grow quite rapidly. Unlike the genetically "normal" tissues of sunflower and other plants, these fragments develop in a disorganized and almost completely patternless manner. Old cultures that have been permitted to form considerable masses of tissue do, it is true, show the beginnings of a radially symmetrical pattern with a considerable center-to-periphery difference in organization, but this is never very far-reaching. Such old cultures have a central core of relatively uniform tissue, made up of moderately large, approximately isodiametric, starch-laden, parenchyma-like cells interspersed with occasional small groups of mostly radially oriented scalariform cells. This is a "mature" tissue, no longer involved in proliferative growth, stable, comparable to the medulla of a complete plant. Around this is a shell of irregular tissue made up largely of areas of rapidly proliferating cells engaged in the fluid transformations of growth and development, unstable, and easily modified in behavior by local differences in impinging stimuli. Interspersed with these meristematic areas and extending over much of the surface of the mass are irregular groups of giant

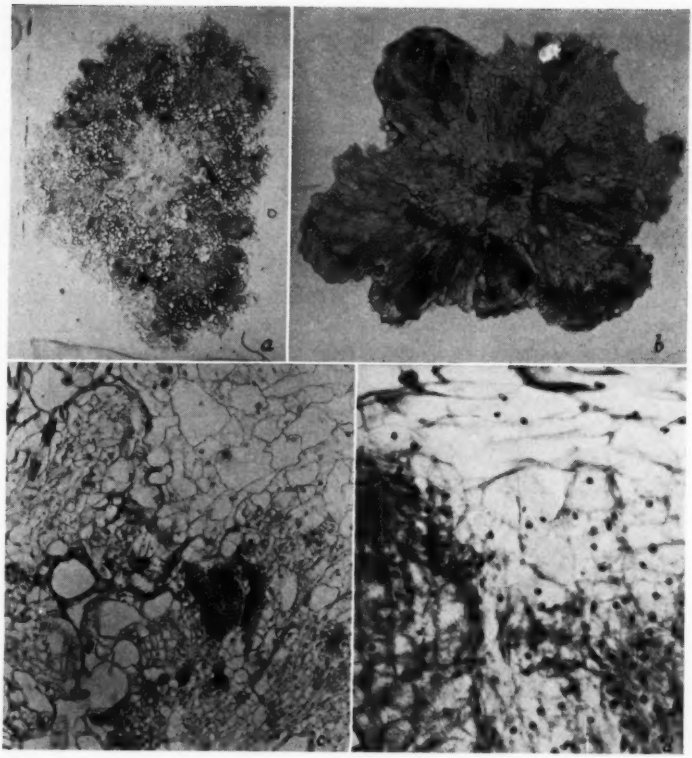

FIGURE 5

"*a*"—cross section through a *Nicotiana* gall culture similar to that shown in Figure 4d., X 10. (*Amer. J. Bot.*, 1939, **26**, 61.) "*b*"—cross section through a sunflower tumor culture similar to that shown in Figure 4e. X 13. "*c*"—detail from the lower potrion of "*a*" showing the irregular distribution of meristematic areas in the outer margins of such culture. X 70. "*d*"—detail of the margin of a culture similar to that pictured in "*b*" showing the meristematic activity concentrated just below the surface of the culture. X 165.

cells or "callus blasen," stable, no longer functional, frequently thickened or necrotic, and roughly comparable to the dead or dying bark of a tree.

Here is indeed a tissue culture, a relatively patternless mass of cells with which to carry out experiments on morphogenetic control. By choosing a material which, due to its peculiar genetic constitution, we

know to be morphogenetically unstable, and then isolating it in a relatively simple unoriented environment, we have eliminated most of the polarity of the tissue. Similar, but slower-growing masses can be obtained from carrot, from wound callus of poplar and willow, etc. By modification of the environment one should, theoretically, be able to control and direct whatever pattern can be evoked from these masses. Whether or not that is actually possible I will discuss later.

The origin of this aberrant behavior is known to be genetic. Beyond that it is no more amenable to experimental analysis than is any other complex manifestation of gene action. Two years ago, however, we found a second type of material which behaves in a somewhat similar fashion, the origin of whose behavior is not genetic but environmental, and thus theoretically capable of more facile experimental manipulation and possible analysis. Although I may seem to be trespassing somewhat on Dr. Riker's territory in this particular case, it fits so well into the continuity of our tissue culture program that, with due apologies to him, I propose to discuss it at some length. I refer to the secondary galls which arise on certain composites, notably the sunflower, following infection with crown-gall bacteria.

If young sunflower plants are inoculated by puncture with a needle dipped in a suspension of a virulent culture of the crown-gall bacterium, *Phytomonas tumefaciens,* there arise at the sites of inoculation rapidly growing, disorganized tumefactions, the tissues of which contain scattered foci of bacterial growth. The overgrowth appears to be the direct result of the presence of the bacteria. Frequently there later arise at some distance from these primary growths smaller, smoother, slower-growing metastatic tumefactions or secondary galls. Unlike the primary growths, these secondary galls do not contain the bacteria and are thus only indirectly brought about by the presence of bacteria, elsewhere in the host. If fragments of sterile tissue are excised from the interior of these secondary galls and placed on nutrient agar, they too grow as tissue cultures which superficially resemble very closely those obtained from the *Nicotiana* hybrid.

Internally the resemblance is less pronounced. Although far from representing the type of growth one obtains in an intact healthy plant, or even in a tissue culture from healthy tissue of the same plant, these masses are not without organization. Active proliferative growth is restricted to a narrow band just under the surface. The interior is made up largely of scalariform cells interspersed with typical parenchyma. The tissues are either oriented in bands radiating from the

center outward or in scattered whorls like the eyes in bird's-eye maple. The organization is loose enough so that the mass is not woody or fibrous but rather of a friable texture. This behavior is universal in cultures from secondary crown-gall tumors and represents a pronounced modification in pattern and a reduction in morphogenetic restraint brought about by some stimulus from the original inoculum of crown-gall bacteria. What this stimulus is we do not yet know. Such masses retain this characteristic behavior indefinitely *in vitro*. They possess in addition another and very important characteristic. When fragments of such cultures are grafted into healthy sunflower plants, they develop into typical crown galls, that is, they retain this acquired characteristic in new hosts just as do scions of the gall-producing hybrid *Nicotiana* when grafted onto gall-less stocks of either parent. The implants in the early stages can readily be identified by the characteristic whorls of scalariform cells, so that the origin of the gall can be traced. It seems to come entirely from the implant and not at all from the host, which is just what we should expect from a non-infectious abnormality. Here again is a material having a greatly reduced complexity of pattern, hence a material theoretically suited to the experimental study of the origin of any pattern which can be evoked therein. In this case it is, in addition, possible to bring about this reduction in pattern at will by known procedures. We also have evidence that similar self-propagating modifications in behavior pattern can sometimes be induced by treatment of healthy plants with certain of the well known growth substances.

Can we, however, evoke new patterns or restore lost patterns in these experimental materials?

It takes time to apply a new experimental technique to specific problems. I have had these *Nicotiana* galls in culture now for five years, the sterile crown galls of sunflower for two. During that time my laboratory has been occupied in clearing up a number of nutritional problems with excised roots. Experimental morphogenesis has until very recently had to take second place. I have, however, been able to do a little, and Dr. Riker, Dr. Burkholder, and Dr. Thimann have verified some few of my observations on material I have been able to furnish them. Dr. Skoog has, I believe, some additional data over and above verification of my results, but that is as yet unpublished and unavailable in detail.

I have one set of evidence, however, which does indicate the feasi-

bility of such experimentation. This arose out of a chance observation made some years ago on *Nicotiana* callus cultures.

Excised roots of tomato, and of most other species, regularly float at or near the surface of a liquid nutrient so that the actively metabolizing tissues are rarely more than a millimeter distant from a liquid-air interface. Occasionally they fail to float, sinking to the bottom of the flask under a layer of 8 to 10 mm. of liquid. Roots that do not float regularly grow poorly. The quality of maintaining itself at or near the interface and the quality of active growth are thus closely correlated. It is not known which of these facts is primary and which secondary. It is conceivable that an actively metabolizing tissue might lay down low specific gravity materials such as fats, or give rise to occluded intercellular gases, which would cause the tissues to float. It is equally possible that tissues of low specific gravity, floating at the surface, might be in a position to obtain more adequate supplies of oxygen, hence be in a position to metabolize more actively and grow more rapidly. We have not clearly determined the causal relationships of these two phenomena. But many observations point to the floating being primary and not secondary. Thus the geotropic response rapidly disappears in cultures immersed in an unaerated nutrient but reappears if the nutrient is aerated. Hoagland and his group have long stressed the necessity for aeration for normal metabolism of root systems. When, therefore, in preparing cultures of procambial tissues, these were observed to sink regularly to the bottom of a liquid nutrient, it was assumed that this condition might be deleterious to their development and a variety of methods for keeping them at the nutrient-air interface was tested. They were supported on absorbent cotton, on glass rods, on pieces of waxed paper supplied with wicks of filter paper, on thoroughly washed agar, etc. All of these methods resulted in a rapid, undifferentiated growth. Nevertheless, cotton and filter paper are likely to supply added growth substances such as thiamin, glass is clumsy to handle, waxes are both clumsy and theoretically capable of supplying either toxic or stimulating solutes. Agar seemed to be the least objectionable supporting medium and has been generally used. It was noted in the course of these studies that cultures which slipped from their supports or were for any reason not supported at the air-liquid interface regularly grew in a very different manner from the others. Instead of the subglobose, glistening-white, unorganized tissue masses characteristic of the well aerated cultures, those growing under a deep liquid layer

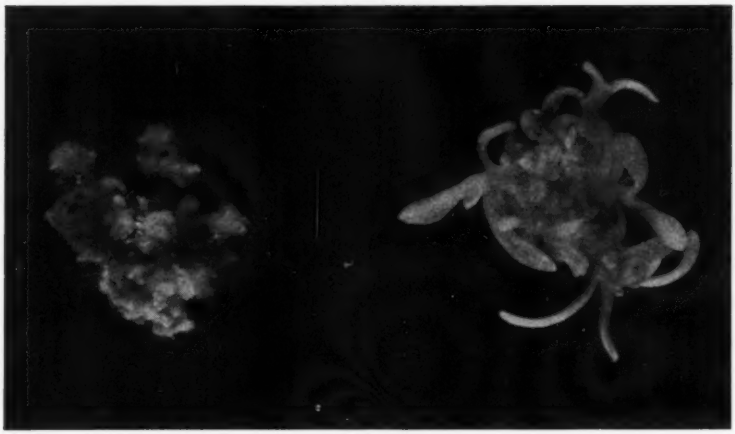

FIGURE 6

CULTURES OF *Nicotiana* GALLS

That shown at the left was maintained continuously on a semi-solid nutrient. That at the right was grown first on a semi-solid nutrient and was then placed in a liquid nutrient where, submerged and cut off from adequate aeration, it responded by forming leaves and stem growing points. X 3.3. (*Bull. Torrey Bot. Club*, 1939, **66**, 509.)

rapidly developed green pigment, their surface layers became organized by well oriented sequences of cell divisions into numerous typical growing points, these developed into short stems with well formed leaves having typical stomata, glandular hairs, and other highly organized members, and there appeared typical plantules. This definite organization was regularly evoked by immersion under a layer of nutrient either in the presence or absence of agar. It is therefore not a reaction to any constituent of the agar as such. And it is completely reversible, the disorganized growth recommencing when leafy cultures are brought into the air, resulting in the gradual distortion, overgrowth, and ultimate disappearance of the differentiated parts.

Here, then, is a well defined morphosis which can be controlled and manipulated at will. We have shown with a fair degree of certainty that the change is not due to any constituent of agar nor is it due to the difference in texture of the liquid and the semi-solid substratum. It seems to be clearly a result of *position* with respect to the interface. The most obvious difference in environment resulting from this transposition is the difference in available oxygen supply. Oxygen is only sparingly soluble in water or in a dilute aqueous solution, and the

gradient in oxygen content downward in an undisturbed aqueous layer is a very steep one. The floating or otherwise elevated cultures are in contact with gaseous air over all of their external surfaces, as well as the surfaces of many of the intercellular spaces which abound in these spongy masses. Only the deepest tissues are cut off from adequate aeration. In immersed cultures, on the other hand, all oxygen available to the tissues has to come from that dissolved in the circumambient liquid, and even the surface layers are cut off from anything but a very meager oxygen supply. If organization, oriented growth pattern, is a response to reduced oxygen supply, we would thus expect morphogenetic differentiation to be most pronounced in immersed cultures and in the interior of exposed cultures. Such actually is the case, for in exposed cultures it is only in the interior that we find organized, scalariform elements formed and find them oriented in a definite fashion, that is, radiating from the center of the culture outward. The whole picture, when taken with what we know about the dependence of metabolism on oxygen supply, strongly suggests that this particular type of morphogenetic response is in fact a response to various degrees of aeration. Our attempts to test this possibility by direct experiment have so far been rather crude and inconclusive.

The conclusion that this morphosis is an aerogenous one would stand upon somewhat firmer ground if sunflower crown-gall tissues behaved in the same way. They do not. But we must remember that these crown-gall tissues, while still sunflower and, so far as we can determine, of normal genetic constitution, have nevertheless been profoundly modified in some as yet unknown manner in their fundamental behavior and that, while in the case of *Nicotiana* gall tissue we are merely re-awakening a behavior pattern which is characteristic of the tissue while still in the intact plant but had disappeared for many cell generations under the optimal conditions of tissue culture, in the case of crown-gall tissue we would be evoking a response which does *not* ordinarily occur in attached crown galls.

I think that the important thing is that we have, in the oxygen-sensitive morphosis of the *Nicotiana* calluses and in the *Phytomonas tumefaciens*-sensitive morphosis of secondary crown-gall tissues of the sunflower, two reactions which can be manipulated under the strictly controllable experimental conditions of tissue cultures. I should like to see these disorganized tissue masses subjected to oriented gradients of oxygen supply as Brachet and Shapiro have done with

echinoderm eggs, to electropotential gradients and oxygen-hydrogen gradients as Lund and Rosene have done with onion roots, to pH gradients and pressure gradients as Whitaker and Kny did with *Fucus* eggs, to mechanical tensions as Weiss has done with animal tissue cultures, etc. I have no doubt that there are many other such manipulable morphoses which can be treated in the same way and that their discovery and analysis in future years may give us a much sounder insight into the causes of the multitudinous variations in form and function which characterize the living creatures among whom we live.

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THE RATES OF GROWTH IN GENITAL TISSUES AND THE HORMONAL REGULATION INVOLVED

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One of the most important factors in growth of tissues and organs is the multiplication of cells by mitosis. In many tissues of the body, cell division occurs throughout life. Sometimes this is a slow but continuous process. In other tissues, cell division may occur in waves interspersed with periods of rest, or of function such as secretion. In the genital tissues especially, these waves of cell division are dependent upon hormonal stimulation. These periods of accelerated cell division in the female genital organs are the chief concern of the present paper.

There are, of course, other important factors in growth besides cell multiplication which must be recognized and set aside from the present discussion in order that the subject may be circumscribed within reasonable bounds. Broadly defined, any increase in size or weight may be classified as growth. Two successive observations are usually required. Factors other than cell division which might contribute would include (*a*) enlargement of cells without cell division, (*b*) increase in fluids either between the cells of a tissue or in cavities or lumina in organs, and (*c*) increase in blood or lymphatic vessels either by true growth or by distention.

When the organs under study are internal organs and operative procedures may influence their growth, it is highly desirable to attempt to visualize rates of growth by a single observation. Sometimes this is difficult if cell division is not rapid at the particular instant the organ is under study. The recent use of the drug colchicine in problems involving cell division has been a great aid in estimating this particular factor in growth (Lits, 1934). This drug holds, or "freezes," cells in mitosis for a period of eight or more hours, and thus accumulates them in an easily recognizable condition so that in one observation an estimate of the rate of cell division can be made. In some rapidly growing organs 30, 40, or even 50 per cent of the total number of cells in a particular tissue may be held in mitosis, the majority in the metaphase. Therefore, the evidence of cell division for considerable inter-

vals is collected for study. It simplifies the work by making counts of cells and computation of mitotic indices unnecessary. Instead, estimates can be made on the basis of + to ++++, so that at a single glance certain contrasts in rates of growth of neighboring tissues are easily apparent. For instance, students of the anterior pituitary have fractionated its hormones into follicle stimulating and interstitial cell stimulating hormones. From the names one might infer that the actions were similar but upon different tissues. When the ovaries are studied by the use of the colchicine technique at different phases of the normal cycle, at the time these hormones are known to be acting, a remarkable contrast is apparent. The cells of the majority of the medium sized follicles are found to be undergoing extremely rapid mitotic division. The surrounding cells of the theca also show many mitoses. The interstitial cells, not far removed from the follicles, contain no mitotic figures. They are completely quiescent as far as cell division is concerned.

When the reaction of the ovary to follicle stimulating hormone, and to interstitial-cell-stimulating hormone is studied in hypophysectomized animals, this contrast is equally clear. Cells of the larger follicles including the theca respond to follicle-stimulating hormone by intense mitotic division. The interstitial-cell-stimulating fraction induces hypertrophy but not hyperplasia in interstitial cells.

Another point emphasized by the use of the colchicine technique is a clear delineation of uneven growth in medium sized follicles which may presage elimination of some during the course of the successful development of a few. It has been recognized for some years that in such animals as the mouse and rat where seven to nine eggs may be ovulated at each estrous period, the eggs which successfully reach ovulation represents a few selected from a considerably larger number. In recent counts in the ovary of the rat an average of 22 follicles per ovary were counted. This would make 44 follicles in two ovaries from which in the course of four days an average of seven to nine follicles might be expected to reach ovulation. There is now experimental evidence that this selective elimination depends upon a restricted amount of pituitary gonadotropin available for the follicles, for if additional pituitary substance is supplied all 44 follicles may be successfully brought to ovulation (Smith and Engle, 1927). Therefore pituitary stimulation is necessary for the development of the follicle during the last four days before ovulation in the mouse and rat.

Previous to this time, i.e., until the follicle attains medium size with beginning of secretion of small amounts of *liquor folliculi*, growth of the follicle may occur evenly in the absence of the pituitary gland. Therefore, up to medium size some inherent ability to grow dominates the division of the follicle cells and theca. In animals killed at estrus, when the ovaries contain a set of preovulation follicles or (if ovulation has occurred) a set of newly forming corpora lutea, the next largest set of follicles can be classified in three groups according to rate of growth when studied with the colchicine technique. At this time (*a*) a majority of follicles are usually growing evenly, that is, mitotic figures are fairly evenly distributed through the follicle and through the theca. Other follicles of similar size may show two types of uneven growth, (*b*) many mitoses around the ovum but few or none in the external layers of follicle cells and theca, or (*c*) many mitoses in the theca with very few or none among the follicle cells. The former type of uneven growth may be interpreted as due to a continued but waning influence from the ovum which still acts upon adjacent follicle cells, but is not strong enough to reach the outer boundaries of the follicle. The predominance of mitoses in the outer parts of the follicle is interpreted as resulting from a stimulus from outside the follicle, probably the follicle-stimulating hormone of pituitary origin, for the large follicles cannot grow without it. This interpretation would require an interaction of both internal and external stimuli for even growth of these medium sized follicles to the point of ovulation (Allen, Thomas, Wilson, and Hession, 1943).

Many instances of interesting stages which seem to confirm this interpretation are to be found in normal ovaries. For instance, ovaries of the rat carefully timed from the onset of estrous behavior give us a milestone from which to time ovulation, which occurs on the average of about 10 hours later. If taken at 16 hours after onset of mating behavior a majority of follicles of the ovulation group have already ruptured. (In a considerable series timed by this method by Boling ovulation occurred at a median of 10 hours and always before 16 hours.) Occasionally in such animals one or two large follicles which had failed to ovulate with their generation still remained in the ovary. The ova may have undergone maturation division and some indication of corona formation and secretion of secondary *liquor folliculi* may be apparent. Follicle cells of the *cumuli* may contain a considerable number of mitotic figures. Sometimes mitoses are present in the *granulosa* forming the base of the cumulus, but absent or very

scarce in the walls of the follicle distant from the ovum. Such follicles usually have few or no mitoses in the theca. This type of follicle is interpreted as still living but doomed for resorption. Some stimulus for mitosis still apparently emanates from the egg. Scarcity or lack of mitoses in the theca and outer granulosa may indicate a lack of pituitary gonadotropin.

Occasionally large follicles are found after ovulation of others of their group in which maturation changes have not occurred in the egg. Such follicles may show a high number of mitoses in the theca and few or none in the *granulosa* and *cumulus*. This type of follicle is interpreted as one in which the stimulus for mitosis from the egg has failed, while pituitary gonadotropic stimulus is still adequate.

This interpretation of two sources of stimuli for mitosis, acting upon the follicle during the last four days of its development in the rodent's ovary, may be used as a basis for prediction concerning the fate of particular follicles. It is probable that the gonadotropic stimulus is necessary not only for continued cell division but also for the secretion of the *liquor folliculi* which distends the follicle and makes ovulation possible. That cell division and secretion go on at the same time in these rapidly growing follicles is supported by many studies of the normal ovary.

In contrast to these two concurrent processes in the follicle, mitotic division and secretion, conditions in the uterine glands are worth comment. During estrus when the uterine glands are secreting, they are usually distended because the secretion is held in the lumen of the uterus by constriction of the cervix. Soon after ovulation this secretion stops. During secretion one seldom, if ever, finds mitotic figures in the glandular epithelium. When studied by the colchicine technique it is noted that the first wave of mitotic division appears in the tips of the glands 24 to 32 hours after the onset of mating behavior of the previous estrus. It was formerly shown that mitosis in the uterine glands is one of the first responses in the ovariectomized animals to injections of estrogen. Therefore in the normal animal this wave of mitosis in the uterine glands must be the first indication of estrogenic secretion leading to the next estrous period more than three days hence. Use of the colchicine technique makes a sharp differentiation of these phases of function possible.

It had formerly been shown that it requires 48 to 54 hours for the full estrous growth of the vaginal epithelium in ovariectomized rodents after a single injection of estrogenic hormone. The greatest

rate of cell division is reached at about 36 hours. At this time cells of the first and second basal layers contain a very great number of mitoses (Allen, Smith, and Gardner, 1936).

When the normal animal at estrus is studied by the colchicine technique it is found that mitotic division is at a very low ebb. In other words the wave of cell division which has produced this thickened epithelium is almost completely spent. Not until 14 to 22 hours after ovulation does an increased incidence of mitosis appear in the vaginal epithelium to start the growth wave for the next cycle. This is coincident with the first appearance of mitosis in the uterine glands. The colchicine technique shows that at these times cells of the surface epithelium, underlying connective tissues and uterine muscle are entirely quiescent as far as cell division is concerned. The wave of cell division reaches the surface epithelium of the uterus some hours after it first appears in the tips of the uterine glands. It appears even later in the stroma.

In the muscle layers of the uterus cell division reaches considerable proportions only after it has stopped in the epithelial tissues. It is highest when the uterus is being distended with secretion at estrus. That distention may also be a stimulus for cell division of the muscle cells has been shown by the insertion of paraffin pellets in the uterus of the ovariectomized rabbit and consequent mitosis in the uterine muscle (Allen, Smith, and Reynolds, 1937). Use of the colchicine technique helps to circumscribe the wave of cell division in the uterine muscle and makes it so easily evident that there can be no question as to an increase in the number of muscle fibers in response to either stimuli from hormones or from mechanical distention. This definity disproves the statement still carried in some textbooks of obstetrics that uterine muscle cells do not divide as the uterus enlarges during pregnancy.

There has been much discussion in the literature about the extent to which the uterine tubes feel the successive cycles of growth and degeneration to which the uterus is subject. In the mouse, during degenerative phases of the cycle, the tubal epithelium, especially the ciliated part, shows degeneration by extrusion of a considerable number of nuclei through the external membrane. This should require cell multiplication for replacement. Use of the colchicine technique shows that mitosis does occur here to a considerable extent, usually at and soon after estrus, but not at other times in the cycle. From 8 to 20 cells in mitosis may be found in a single section of the fimbriated

end of the uterine tube of the mouse. Mitotic division of epithelial cells occurs also to a considerable extent in the fimbriated end of the uterine tube in the rat and monkey. It can be induced by estrogenic stimulation in ovariectomized animals. Even with the colchicine technique, however, it is extremely difficult to find mitotic figures in the non-ciliated portion of the uterine tubes. These cells must have a longer life and be less subject to degeneration.

A remarkable contrast in rates of growth in adjacent tissues is seen at the uterotubal junction in animals stimulated by estrogenic hormone. The uterine epithelium at this time may have as many as 30 per cent of its cells in mitotic division, and this high incidence of mitosis extends to the epthelium covering the fold at the entrance of the tube into the uterine lumen. Although there is no transition in type of epithelium in the proximal end of the tube, the tubal epithelium is devoid of mitotic figures. There is a sharp line of demarcation at this point. As far as I am aware this was not fully appreciated until it had been studied with the colchicine technique.

While studying sections of the lower vagina we have routinely studied the lower rectum and frequently sections of hairs from the skin and parts of the preputial glands have been included. Glands of the rectum reflect the success of the colchicine technique,—the lower one-third or one half of the gland may have an extremely high incidence of mitotic division. One seldom, if ever, finds mitotic figures in the outer parts of the glands. The interpretation of replacement of rectal epithelium and a shifting of the younger cells from the tips of the glands to the outer surface is clearly indicated. While this has been known for a long time, the localization of cell division is surprisingly accentuated by this technique. The growth of hair has been studied extensively by Danforth, Trotter, and others. Danforth clearly showed that adjacent hair follicles had varying rates of growth, that is, that certain follicles might be growing rapidly while adjacent ones were quiescent. This may not be entirely true of the hair of animals where the hair is shed and regenerated seasonally. The colchicine technique frequently discloses certain follicles to be literally packed with cells in mitosis, while adjacent ones are devoid of mitotic figures.

The preputial glands in rodents are sebaceous in type and apparently subject to phases of secretion alternating with phases of intense mitosis. It is probable that these are under hormonal control, and that the mitotic phase reflects an increased estrogenic level, for at

certain times corresponding with proestrus and estrus, the preputial glands have an extremely high incidence of mitosis.

The mammary glands also react to estrogenic stimulation by extremely high incidence of mitosis in both ducts and alveoli. At the periphery of regions of growth, alveoli are found which contain many mitoses while the older branches of the mammary tree are quiescent as far as cell division is concerned. Remarkable proliferation of the crop gland in pigeons is induced by injections of lactogenic pituitary hormone. The histologic picture following treatment with colchicine reveals an almost unbelievably intense mitotic division of this epithelium (Leblond and Allen, 1937).

When one studies the decidual reaction with the colchicine technique the very first evidence of mitosis among the connective tissue cells underlying the epithelium can be noted. In the mouse it usually occurs on the fourth day after fertilization when the segmenting ova have entered the uterus. Mitosis at this time is restricted to the subepithelial zone. The next day it extends further toward the muscle layers. By the sixth day the wave of mitosis has reached the inner circular muscle layer, and mitoses are absent from the sup-epithelial zone. Thus cell division spreads centrifugally like the waves when a pebble is tossed into a pond.

At several times in both the non-pregnant cycle and during pregnancy there is an extremely rapid growth of blood vessels, first around the growing ovarian follicle, and second, in the development of a corpus luteum following ovulation as the former granulosa is transformed into luteal tissue, and third, in the formation of the vascular zone of the decidua after the 8th day of pregnancy (in rodents). Use of the colchicine technique sometimes shows as many as three or four mitotic figures in a single high power field visible in the endothelium sprouts of these vessels.

The segmenting ovum is known to have a very rapid rate of cell division. We were further greatly surprised to find a morula in the 32-cell stage in which 19 cells were in mitosis. Later as histogenesis continues in the developing embryos, a differential rate of growth is clearly apparent with the colchicine technique.

There is evidence for the formation of new eggs from undifferentiated cells of the germinal epithelium in the ovaries of small mammals. This epithelium is subject to waves of proliferation which provide (*a*) increased numbers of germinal epithelial cells, (*b*) additional ova and (*c*) perhaps also follicle cells (Allen and Creadick, 1937; Schmidt,

1942). In the mouse and guinea pig considerable numbers of mitoses can be found by the use of the colchicine technique. In some of the other small laboratory animals evidence of formation of new eggs is present but rests upon finding invagination of cell cords from the germinal epithelium. There must, however, be proliferation of these cells to replace those which are included in the invaginations. It is probable that ovogenesis is synchronized with the estrous or post estrous phases of the cycle. At these times the ovaries are very vascular and active metabolically.

Proliferation of tissues of the uterus and vagina depend almost entirely upon ovarian hormonal stimulation. They do respond by proliferation to other stimuli, but normally the growth is called forth by hormonal stimulation. Fortunately ovarian hormones flow and ebb alternately; retrogression follows growth and returns the organs to relatively small size in the absence of pregnancy. Following fertilization a long period of growth of the uterus during pregnancy overrides these shorter cyclic fluctuations.

Cancer of the genital system usually involves far longer periods of growth. Therefore, attempts were made to induce cancer of the *cervix uteri* by continued estrogenic stimulation without permitting periods for retrogression. Mice were injected two or three times weekly with fairly high doses of estrogen without permitting intervals of rest. The dose proved toxic to some strains of mice. Other more hardy strains stood the estrogenic treatment of this level without serious inconvenience. Of the mice which survived the chronic estrogenic treatment for more than one year, 50 per cent or more developed cancer of the *cervix uteri* (Gardner and Allen, 1934; and Allen and Gardner, 1941). The cancers usually could not be recognized until serial sections were made after death. Four stages in the development of large malignant tumors were obtained. They varied from an anaplastic growth, usually beginning near the posterior lip of the cervix, to increasingly large areas of epithelium infiltrating into the sub-adjacent connective tissue. The larger tumors filled the pelves and prolapsed from the vaginae. Cancerous tissue also grew into the muscles of the rectum and bladder. Metastases to regional lymph nodes occurred in some instances. Transplants from regions of the tumor which were free from infection grew in other animals of the same strain, males as well as females, and it was not necessary to continue injections of estrogen into the hosts. Therefore this experimentally induced carcinoma had attained the ability for autonomous growth.

The more advanced lesions were lethal, perhaps from pressure on urethra and rectum.

It is not known at what stage of the experimental procedure the growth of the cervical epithelium becomes pathologic. Surely for many months it is stimulated to outward growth without any cancerous characteristics. Why in 50 per cent of the animals of a group cancer should develop while in the other 50 per cent of the same group growth should continue to be external and slough off, there seems to be no clue at present. It does seem probable, however, that the same hormone which stimulates normal growth when used for short periods can, when injected chronically without permitting periods of regression, induce abnormal growths which are pathologic and lethal.

Recently a conference was held which attempted to gather together all information regarding various types of tumors which could be induced experimentally by administration of hormones. Tumors associated with upsets in endocrine function were also summarized. Abstracts of these papers showed a considerable range of cases in which there is evidence of endocrine complications (11). Therefore hormones as stimuli or inductors of normal growth must be followed into the field of pathologic growth.

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CALCIFICATION AND OSSIFICATION

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This paper is a review of the results of a program carried out during the past five years in collaboration with Dr. William Bloom and others, in an attempt to bridge some of the gaps between the chemical and morphological approaches to the study of calcification. The work has led us into nearly every phase of the physiology of bone, but the emphasis in this paper is on the more purely biological aspects of the work, with special reference to growth and regeneration of bone. The treatment is of bone as a tissue, with little reference to the function of the skeleton as a whole, or to the shape, adaptation, or development of individual bones.

Much of the work described was made possible by an improved histological routine (1) including an adaptation of the von Kóssa method, which permits positive staining of bone salt in thin serial sections of undecalcified bone, and simultaneous observation of deposits of this salt and of the cellular elements of bone (Figure 1). In the embryonic rat (2), in most bones, the periosteal bone is laid down and there is extensive calcification in the cartilage model before there is any penetration by blood vessels. In the rapidly growing long bones, erosion and invasion at the epiphyseal line may at times almost outstrip calcification of the cartilage. In embryonic rats and in young rats, puppies and kittens (1) calcifiability is conferred upon cartilage matrix as the adjacent cells become hypertrophic, and in the presence of favorable humoral conditions calcification occurs at this time. Bone matrix, however, is calcifiable as it is laid down, and calcification, under optimum conditions, occurs simultaneously with deposition of the organic matrix. The concept of "physiological osteoid," or of uncalcified osseous tissue, in the sense of a necessary stage in the formation of bone, is thus not valid. Local areas of osteoid borders on calcified bone may, however, occur under physiological conditions, and are attributed to failure in the local supply of bone minerals. In the puppy the occurrence of such borders (Figure 2) is increased when vitamin *D* is omitted from a diet adequate in other respects (3).

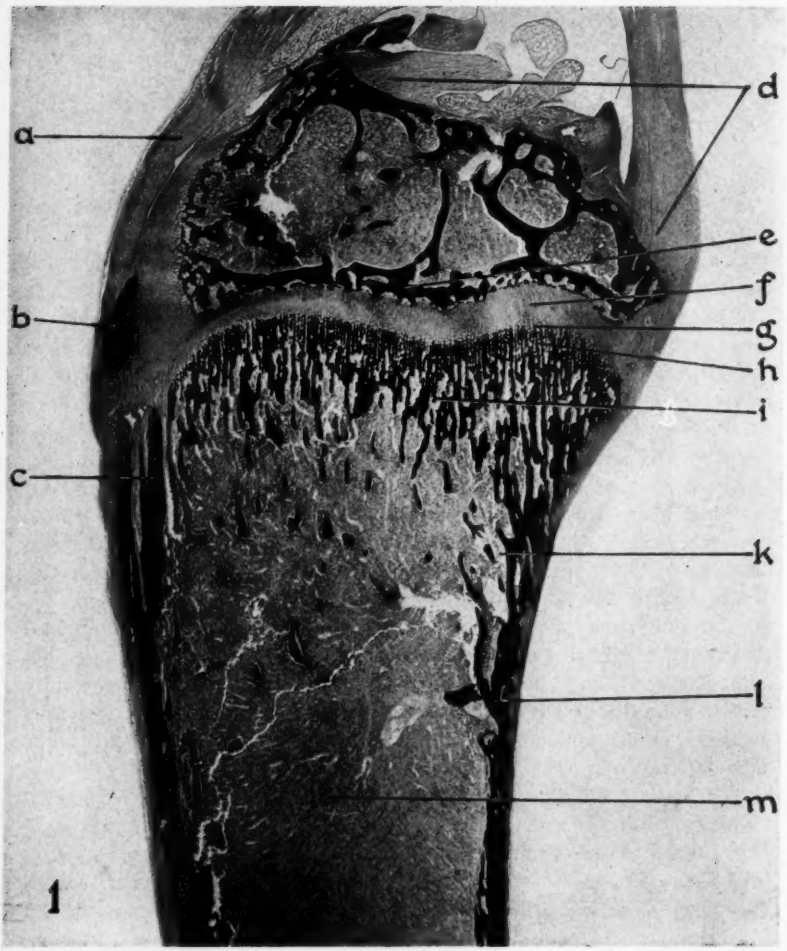

FIGURE 1

SAGITTAL SECTION OF HEAD OF UNDECALCIFIED TIBIA OF NORMAL RAT, AGE SEVEN WEEKS, WEANED TO BILLS' STOCK DIET AT THREE WEEKS

(*a*) Patellar tendon; (*b*) ossification center of anterior tibial tubercle; (*c*) intratendinous ossification in insertion of patellar tendon; (*d*) insertions of crucial ligaments; (*e*) area in epiphysis where osteoid is frequently seen; (*f*) epiphyseal cartilage plate; (*g*) zone of hypertrophic cartilage and of provisional calcification; (*h*) primary spongiosa; (*i*) secondary spongiosa; (*k*) area at junction of spongiosa with shaft where osteoid is frequently seen; (*l*) shaft; (*m*) bone marrow. Formalin fixation, 10 μ, silver nitrate-hematoxylin-eosin. Photomicrograph. x 18.

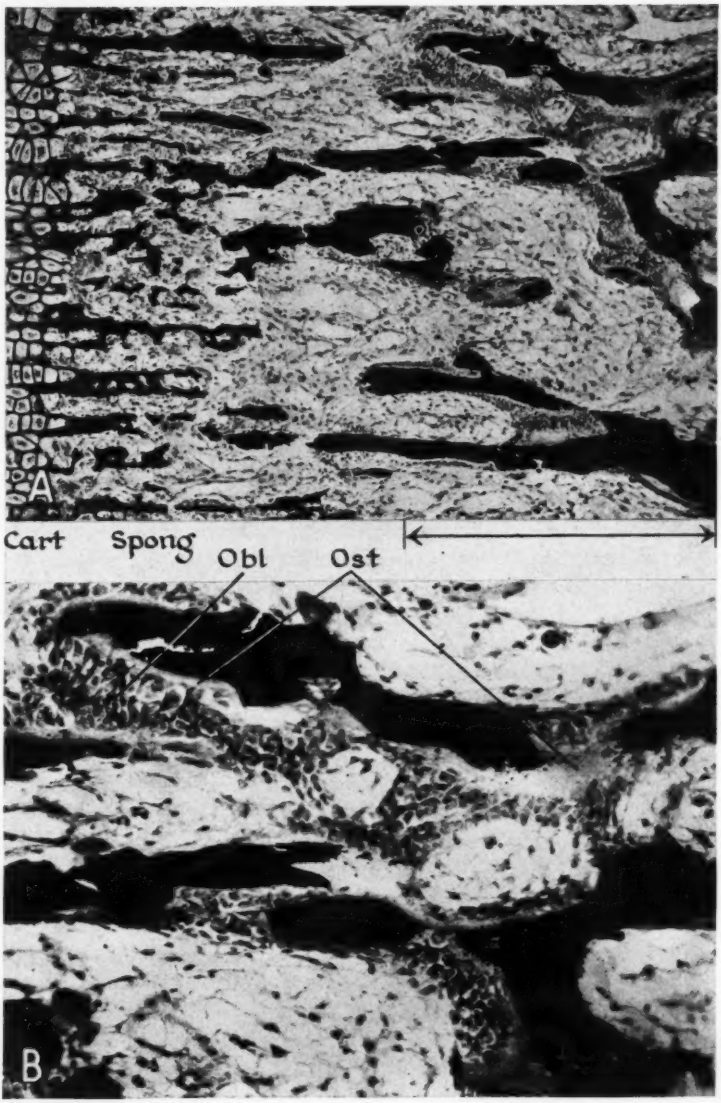

FIGURE 2

A, photomicrograph (x 88.5) of an undecalcified longitudinal section through a costochondral junction of a puppy fed a diet adequate in minerals but without vitamin D; stained with silver nitrate, hematoxylin and eosin. *Cart* indicates cartilage and *Spong*, substantia spongiosa; vertical lines and double arrow indicate area of *B*.

B, higher power photomicrograph (x 198) of area indicated in *A; Ost*, osteoid borders on trabeculae of spongy bone; *Obl*, osteoblasts.

Evelyn Smith Ross has found (4) that the growth hormone of the anterior pituitary, when administered to rats during the weight plateau at the age of six months or shortly thereafter, induces histologically recognizable evidences of active growth in quiescent or lapsed epiphyseal cartilage plates and the adjacent spongiosa. She has also found (5) that rats fed sufficient thyroid substance to produce severe hyperthyroidism failed to show decalcification or failure of calcification of their bones, but premature cessation or marked retardation of growth at the epiphyseal junction did occur.

Growth and regeneration of bone have been studied in relation to tissue cultures and to fractures. Heinen (6) has implanted outgrowths from tissue cultures of the bones of young rats to the anterior chamber of the eyes of the same species. The grafts became vascularized and a chondro-osseous tissue developed. Primitive bone marrow then appeared, and there was invasion and replacement of the earlier tissue by true bone. Finally, a disc-shaped bone, adapted to the size and shape of the anterior chamber, developed, with central marrow, usually fatty, and further growth did not occur. In rats on adequate diets the new bone calcified normally, in rats on diets low in phosphorus and vitamin *D* the intra-ocular bone was rachitic. Heinen also succeeded, for the first time with mammalian tissue, in demonstrating differentiation of bone, ***in vitro,*** from the outgrowth of cultures of bones of rats.

Urist (7, 8, 9, 10) has made an extensive study of the healing of fractures, both experimental and clinical, with special reference to the relation of calcification in the callus to the healing process. The processes of callus formation and calcification of the callus ordinarily occur simultaneously, but may be separated under certain conditions.

After an initial reaction to the trauma of a fracture, the healing process begins. It consists essentially (7) of formation of a fibrocartilaginous callus, originating within the fracture gap, and of formation of new bone, originating at some distance from the fracture line and invading and replacing the fibrocartilaginous callus from without (Figure 3). Whether or not cells arising from the ordinary connective tissue of the callus have osteogenic potencies, cells arising from the deep or cambium layer of the periosteum and from endosteum play the greater part in the formation of new bone. Bone seldom, if ever, arises from within the callus. The process as a whole is an excellent

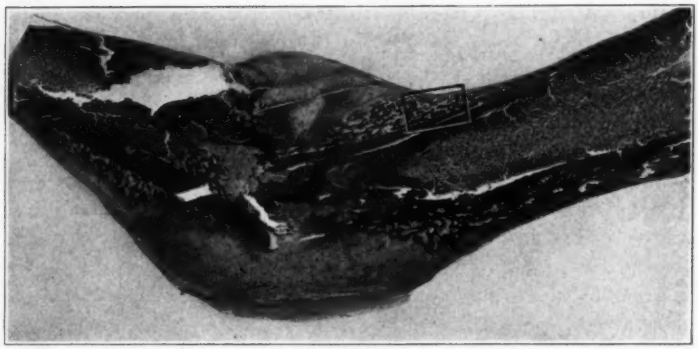

FIGURE 3

PHOTOMICROGRAPH (X 8) OF LONGITUDINAL SECTION THROUGH FRACTURE OF UNDECALCIFIED TIBIA OF RAT AFTER SIX DAYS OF HEALING

Subperiosteal and subendosteal new bone are in contact with fibrocartilaginous callus, which is everywhere uncalcified except at points of contact with new bone. Silver nitrate-hematoxylin-eosin.

example of organization of diverse means to a common end—the nature of the organization process itself is but little understood.

The early stages of this healing process are but little affected by the conditions which will lead to or prevent calcification (8). In the rat, when a rachitogenic diet is fed, the effects of failure of calcification begin to manifest themselves by about the fourth or fifth day following a fracture, at which time invasion of the fibrocartilaginous callus is under way under ordinary conditions. In the absence of calcification this invasion is delayed, the delay being comparable to the delay in initiation of the rachitic type of cartilage removal in the epiphyseal cartilage following the onset of rickets. Healing of fractures in the rachitic rat may be further complicated by encapsulation of the callus cartilage in a dense mass of fibrous tissue or fibrocartilage. Eventually, in the rat, even in severe rickets, there is spontaneous calcification of the callus, leading to union of the fracture by partially calcified osteoid. All of the effects of non-calcification may be overcome by providing a diet favorable to the deposition of bone mineral.

In fractures in the rat, when maintained on an adequate diet, calcification in the intramembranous bone of the callus ordinarily occurs as rapidly as the matrix is laid down (7), as is the case in growing bone (1). In both cases, however, a lag may occur, attributed to local failure in mineral transport rather than to non-calcifiability

of the matrix. In man (10) the lag appears to be the rule, rather than the exception, and is attributed to a rate of osteogenesis greater than the rate at which the calcification mechanism can operate, at the low level of serum phosphate in all individuals except infants and very young children.

The matrix of hyaline cartilage, in the callus in the rat, becomes calcifiable when the adjacent cartilage cells become vesicular or hypertrophic, but only matrix in contact with the invading bone calcifies (7). If the matrix has not been made calcifiable by hypertrophy of the adjacent cells, it calcifies only when converted into bone matrix by the advancing osteogenic process. Only tissues recognizable as bone matrix or cartilage matrix calcify in the callus; there is no random calcification in the fibrocartilaginous callus.

It has been contended that the chief source of mineral for the repair of fractures is from the dead bone at the site of the injury. Urist's studies on the rôle of the local transfer of bone salt in rats (9), however, have led him to the conclusion that the humoral source of calcium and phosphorus is essential for the normal progress of healing, and cannot be entirely replaced by local sources of mineral without considerable delay in the healing of fractures. Similarly, his studies of the healing of fractures in man (10) indicate that bone salt is transferred from the shaft to the callus during rarefaction of the callus-enclosed shaft, but that this process provides only a small contribution to the total calcium deposit within the callus. The studies on man also reveal that there is no consistent elevation in the serum calcium, serum phosphate or serum phosphatase levels during the healing of fractures.

An extraordinarily interesting phenomenon, first described by Kyes and Potter in 1934 (11), has been studied. This is the development of a secondary system of bone, in the marrow spaces of the long bones of birds, now commonly referred to as "medullary bone," and serving the function of storage of calcium to be required for the egg shell (Figure 4). The phenomenon is apparently common to all birds, but its cyclic characteristics are best studied in such birds as the pigeon, in which the egg-laying cycle is brief.

Because of the rapidity of the changes in the medullary bone the material from the female pigeon offers unusual opportunities for the study of bone-forming potentialities of reticular cells, and of the origin and fate of cells associated with the formation and destruction of bone, and has been made use of from this point of view (12). In the pre-ovulatory bone-forming period reticular cells become os-

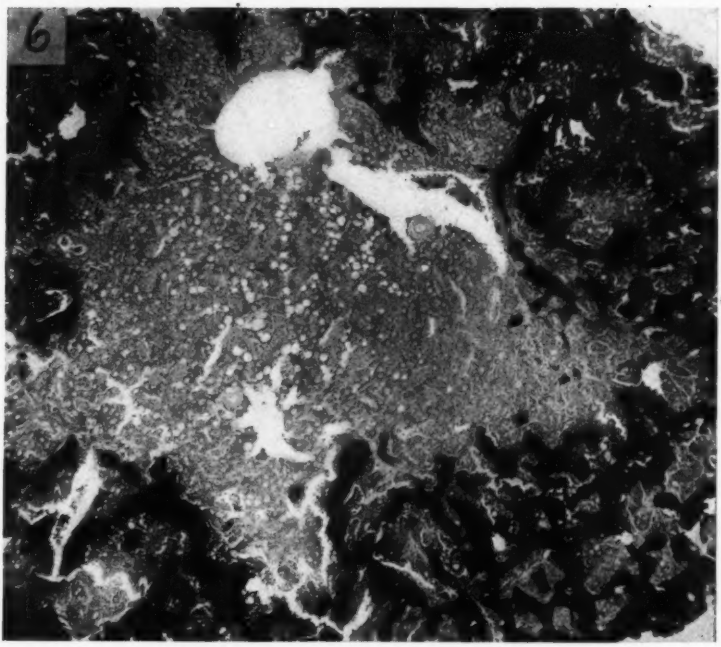

FIGURE 4

PHOTOMICROGRAPH OF SECTION OF UNDECALCIFIED FEMORAL MARROW OF PIGEON WITH 6 MM. FOLLICLE IN OVARY

The pale staining central core of hematopoietic marrow is in sharp contrast to the black trabeculae of medullary bone. Hematoxylin-eosin-silver nitrate. x 45.

teoblasts, and these in turn become osteocytes. In the bone-destroying phase after ovulation of the first egg, osteoblasts and liberated osteocytes become osteoclasts. The latter may turn into osteoblasts during resumption of bone formation. In the post-ovulation period, osteoblasts and osteoclasts again become reticular cells.

There is a considerable body of literature on the production of medullary bone by the administration of estrogens to chickens, pigeons, ducks, and sparrows. We have shown (13) that the ability of the male pigeon to produce medullary bone, under the influence of estrogens, is seasonal, and that the seasonal effect apparently depends upon the fact that androgens, as well as estrogens, are necessary in this species. That the mechanisms are not entirely explained by the

action of estrogens is indicated by the fact that experimentally induced growth of medullary bone is neither as rapid nor as extensive as in the normal cycle in the female, and by the fact that hypercalcemia and medullary bone can be produced together or independently, in the pigeon, according to the experimental conditions (13).

During calcification of the egg shell the process in the bone marrow is rapidly and sharply reversed, with extensive and rapid dissolution of the medullary bone, accompanied by large numbers of osteoclasts (12). The mechanism by which this reversal is mediated, although strongly suggestive of hormonal influences, remains unexplained. There is no evidence that the parathyroid hormone is concerned (13). A similar dissolution of medullary bone occurs with termination of the egg-laying cycle, when the ovary returns to the resting condition.

The bearing of these observations on birds upon the relationship of sex hormones to the bones of mammals has not been wholly clarified, and there are apparently species differences. In the mouse, medullary bone of endosteal origin has been described (14). In the rat we have observed (15), as have others, a condition superficially resembling that in the bird, but the increase in spongy bone is in the region of the epiphysis and is due to a failure of resorption of the spongiosa during the growth in length of the long bones.

In mammals the function of storage of calcium is carried on chiefly by the spongy bone just referred to, near the epiphyses of the long bones. From there calcium is liberated by resorption of small amounts of bone, under the mediation of the parathyroid hormone. There is no evidence that that latter influences the deposition of calcium.

The concept of an "optimum," as opposed to an "adequate" intake of calcium has been emphasized; when the intake is increased over a barely adequate amount, much of the additional calcium is retained in the organism (16). Feeding of added calcium leads to a failure of resorption of the spongy bone (17) and consequently to an increase in the total amount of such bone—an effect similar to that produced by the administration of estrogens. It appears that the organism has a means of conserving amounts of calcium not immediately needed, and that the storage of this element is under the influence of intake, parathyroid hormone and estrogens, as well as of vitamin *D*.

Rickets is essentially a failure of calcification to keep pace with osteogenesis, resulting in the formation of uncalcified osseous tissue, or osteoid, and in a breakdown of the orderly processes of growth at the junction of the shaft with the epiphyseal cartilage. It is our opinion that all of the changes in the bones in rickets are secondary

to the failure of calcification, and that vitamin *D*, in antirachitic doses, has no direct or local effect upon the calcification mechanism in bone. Rickets may result from a deficiency of vitamin *D*, or phosphate, or of calcium. In the latter case, however, the picture is atypical, owing to the occurrence of a secondary hyperparathyroidism (17).

Large doses of certain activated sterols, including vitamin *D*, induce marked changes in bone (18), somewhat resembling those produced by parathyroid extract, and associated with hypercalcemia. This action may be dissociated from the anti-rachitic action of vitamin *D*, since dihydrotachysterol, which is only slightly anti-rachitic, exhibits the calcemic effect and the effects upon bone.

The rôle of phosphatase in calcification still remains to be clarified. We have found (3) that phosphatase is associated with the layer of osteoblasts applied to the surface of growing bone, the enzyme not being found in the matrix of osteoid tissue awaiting calcification. The significance of this finding is still obscure.

The nature of the "local mechanism," which determines whether and where calcification will occur, in the presence of favorable humoral conditions, has been investigated (19). The problem was approached with the working hypothesis that the mechanism is enzymatic in nature, this point of view being supported by the observation that heating to 60° C. causes a complete and irreversible inhibition of calcification of rachitic cartilage *in vitro*. Other influences, including iodoacetate, fluorine, alcohol, acetone, chloroform, drying over phosphorus pentoxide, and leaching in phosphorus-free saline solutions, cause a partial inhibition, usually easily overcome by adding an excess of phosphate to the solutions used. A search for a method of producing a satisfactory reversible inhibition of the local factor, with which the nature of the process could be further investigated, has so far failed.

Others have called attention to a correlation between the appearance of glycogen in hypertrophic cartilage cells and the calcifiability of the cartilage matrix. Our efforts to show that the calcifiability is dependent upon anaerobic glycolysis in the tissue have failed. Glycolysis may be inhibited, to the extent of 70 to 90 per cent, by iodoacetate or fluoride, and this inhibition is accompanied by a slight reduction in calcification, but the latter is easily overcome by adding slightly more phosphate. Moreover, glycolysis, in the absence of inhibiting agents, proceeds at the same rate, whether or not the incubating medium contains enough phosphate to induce calcification in the cartilage matrix. Addition of adenosine triphosphate and of creatine to the

incubating medium has no effect upon calcification. Addition of glutathione and of thioglycolic acid, both of which are reported to inhibit phosphatase activity, still gives excellent calcification in the presence of a phosphate ester and in the absence of inorganic phosphate.

Our work on the chemistry of calcification (20) will not be reviewed here. It is of interest, however, that the local mechanism is operative *in vitro,* in the presence of adequate concentrations of calcium and phosphate, over a range of at least pH 5.7 to 8.7, a remarkable range for a biological process.

We have given intensive study to the problem of the mode of action of the parathyroid hormone upon bone. When very large doses of parathyroid extract are administered to growing rats (21) many of the osteoblasts die; the majority however, change into spindle-shaped cells or osteoclasts, or become phagocytic. There are also extensive necrotic changes in the marrow, recovery occuring by intramembranous formation of new bone, accompanied by development of large numbers of active osteoblasts from the fibroblasts of the scar. Of general biological interest is the evidence that osteoblasts are not permanently differentiated cells, for they change into phagocytes, osteoclasts and reticular cells; moreover, they arise from reticular cells in the marrow and then are associated with the formation of new bone.

Whether bone gives up its mineral to the blood, under the influence of parathyroid hormone, as a result of a direct action of the hormone on the cellular elements of bone, or whether the action is indirect, by way of the kidney, or otherwise, remains unsettled. One of the originators of the first school of thought has transferred to the second (22), basing his defection upon his failure, in common with that of others, to induce hypercalcemia in nephrectomized animals, by administration of parathyroid extract, while one of the originators of the second school, who still adheres to it, recognizes a direct effect of the parathyroid upon bone, in extreme cases. He would call the effects just described, for example, those of "parathyroid poisoning" or "hyperhyperparathyroidism."

We adhere to the belief that the parathyroid hormone acts directly upon the cellular elements of bone, in such a way as to promote the resorption of bone. It is generally assumed, although not proved, that osteoclasts are in some way responsible for the resorption of bone. From this to the assumption that they resorb bone by phagocytosing it is but a short step, but there is little evidence for such a mechanism. To those who adhere to the belief that bone is decalci-

fied, under the influence of the parathyroid hormone, by an increase in the solubility of the bone salt in the circulating fluids of the body, resorption of the organic matrix of bone is a reaction to decalcification.

We have accumulated evidence on these points under a variety of conditions (23) under all of which it appears that the body has access to the calcium salts in bone only by destruction of the bone itself, including the matrix. It thus appears that decalcification of bone is a misnomer, and that the concept of destruction of bone by resorption, involving simultaneous dissolution of both its organic and inorganic

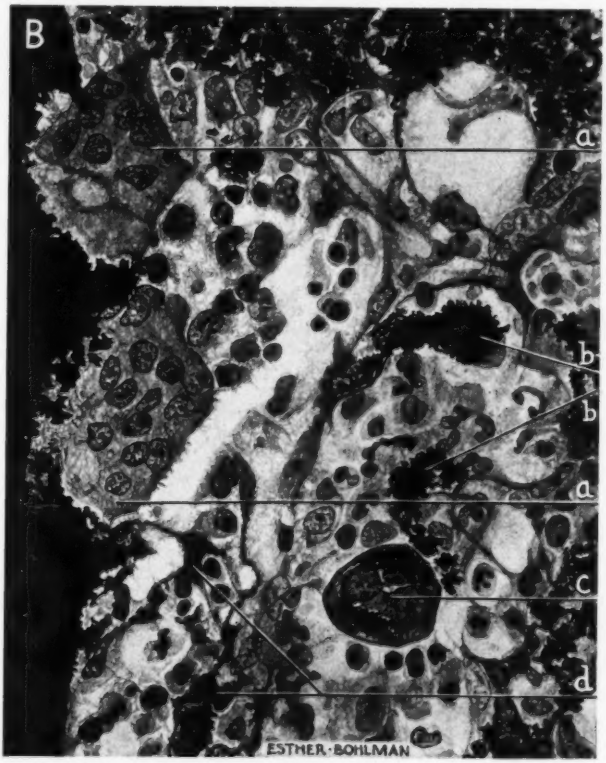

FIGURE 5

UNDECALCIFIED SECTION THROUGH RIB OF PUPPY 24 HOURS AFTER INJECTION OF 250 UNITS OF PARATHYROID EXTRACT PER KILOGRAM OF BODY WEIGHT

(*a*) Osteoclasts, free from aggregated bone salt; (*b*) macrophages, packed with bone salt; (*c*) megakaryocyte, containing bone salt; (*d*) basophil network. Camera lucida, x 681.

constituents, must be substituted for that of decalcification. What is frequently called decalcification of bone is probably failure of calcification of new osseous tissue, formed in the growth or physiological turnover of bone.

Nor have we found any evidence that resorption of bone is accompanied by phagocytosis by osteoclasts. On the contrary, under conditions leading to extensive phagocytosis of the bone salt and of necrotic debris, by macrophages and other cellular elements of the marrow, the osteoclasts remain singularly free from this material (Figure 5).

The work as reported indicates how heavily the physiology of bone leans upon histological methods and points of view. The chemical point of view is not adequately represented in this review, but it is my belief that the immediate outlook for advance in the physiology of bone depends upon close coördination of the histophysiological and the chemical approaches.

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TISSUE RESPONSES TO VITAMIN DEFICIENCIES

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It seems paradoxical that a subject so fundamentally involved in problems of growth, development, and function as nutrition should remain until recent years of secondary interest to biologists in general. The synthesizing capacities of living systems, their adjustability to environmental changes including food, and their capacity to thrive on what were thought to be simple nutrients long diverted attention from the real situation.

We now know, chemically speaking, that the nutritional requirements for even the simple organisms are rather complex, that they vary both qualitatively and quantitatively with the species, and that either mild or drastic changes may be brought about in growth, development, and well being by alterations in the food.

The present rapid development in this field is due to the realization that although a comparatively few substances such as fats, carbohydrates, proteins, and minerals constitute most of the mass of a diet, small amounts of many other substances (vitamins) are also required. The vitamins are naturally occurring organic substances which possess unique molecular structures that the organism is unable to synthesize but which are essential components of the chemical machinery of the cells. Since the organism cannot synthesize these particular molecular configurations from other substances they must be supplied preformed in the food. A number of these substances have been isolated from natural sources and many of them subsequently synthesized. There are others yet to be discovered and isolated.

The biological chemist is rapidly uncovering some of the particular chemical mechanisms in which the vitamins function and the pathologist is learning something of the morphological alterations which follow the interruption of the cell processes in which they are involved. As these two opposite types of advancement approach each other the relation of cell chemistry to morphology should become more evident.

It is the purpose of this short paper to call to your attention some of the morphological alterations subsequent to vitamin deficiencies.

Since Dr. S. B. Wolbach and the author (1) recently published an extensive review of the subject I refer you to that for a bibliography and hence to the original literature for illustrations. Most of what I have to describe comes from the work of Dr. Wolbach and his colleagues at Harvard, including Drs. Howe, Boyle, Swank, and the author. Much of the text is from the above mentioned review.

The morphological changes due to a vitamin deficiency may be peculiar to one type of cell, or, more generally, distributed in several tissues depending upon the particular biochemical process affected and the importance of the process to the cell type. One must endeavor to distinguish between primary effects (the immediate consequences of a deficiency upon tissues in which the vitamin is operative) and the secondary effects (the effects upon the organism as a whole or some other tissue as a consequence of the loss of function of the tissue primarily affected). It is also important not to confuse specific lesions with manifestations of inanition, an almost invariable consequence of any of the deficiencies. Other factors which may affect the morphological picture are degree and duration of the deficiency, and the state of other biological processes occurring simultaneously, i.e., growth, other deficiencies, infection, etc.

Vitamin *A*

Vitamin *A* deficiency produces marked changes in certain types of epithelial structures and in the skeletal and nervous systems. While there are also effects upon the hematopoietic tissues, there is no proof that these are different from the inanitious effects of several other deficiencies.

1. *Epithelial Tissues*

The changes produced in many epithelial structures are to be regarded as the most characteristic consequence of vitamin *A* deficiency because they appear regardless of age and presumably in all vertebrates, though demonstrated as yet only in man, monkeys, cattle, swine, dogs, rabbits, guinea pigs, rats, mice, and fowls. The sequence of the epithelial effect may be epitomized as follows: atrophy of the epithelium concerned, reparative proliferation of basal cells, and growth and differentiation of the new products into a stratified keratinizing epithelium. Regardless of the original function and structure of the region, this replacement epithelium is identical in all locations and comparable in all its layers with epidermis.

The order of the response of various organs varies somewhat with different species of animals but essentially the same organs are involved. The keratinizing metaplasia is found in: (*a*) salivary grands, including the submaxillary, parotid and all accessory glands of the buccal cavity, tongue, and pharynx; (*b*) the respiratory tract, including the nares, maxillary sinuses, Jacobson's organ, trachea and bronchi; (*c*) genito-urinary tract, including the renal pelvis, ureters, bladder, epididymis, prostate, seminal vesicles, coagulating glands, uterus, oviducts, and accessory sex glands of the vulva; and (*d*) eyes and paraocular glands, including the corneal and palpebral conjunctivae, the Harderian, intra-orbital and extra-orbital, and the Meibomian glands. In man, hyperkeratotic lesions of the skin centering about hair follicles may occur in the deficiency after puberty.

The mucosa of the stomach and intestines and the renal tubules do not undergo the above described changes. At most, some degree of atrophy of the intestinal glands may be attributed to the deficiency.

In general, in vitamin *A* deficiency the epitheliums which atrophy and which become replaced by stratified keratinizing epithelium are those which have a secreting (chemical) function in addition to the rôle of a covering layer and whose functioning cells are without power to divide. Repair, therefore, takes place from focally distributed basal cells which multiply, spread beneath the original epithelium, and finally, through coalescence of areas thus produced, form a continuous epidermis-like layer. On the other hand, epithelial cells with chemical rôles, as liver and renal tubules, which do have the power of dividing, do not exhibit marked degrees of atrophy nor are they replaced by keratinizing epithelium. Certain stratified epithelium—cornea, renal pelves, ureters, and bladder—in vitamin *A* deficiency increase their growth rate and become hyperkeratotic, which might be interpreted as evidence that these epitheliums normally have specialized functions which are inhibited by the deficiency. No satisfactory explanation has been found for the fact that the reparative activities of basal cells of many different epitheliums in vitamin *A* deficiency end in an identical stratified keratinizing epithelial product. A natural assumption is that vitamin *A* is necessary in some chemical process uniquely related to normal differentiation and life of this type of cell.

In recovery following vitamin *A* administration, in spite of the common morphology, the epithelium of each region returns to its normal type in morphology and function. The recovery sequences

have been followed by Wolbach and Howe and some of their conclusions are: "All cells of the lowermost layer of the replacement epithelium have proliferative powers as in the *stratum germinativum* of epidermis"; "The important histological features of repair involve removal of the layers of cells irreversibly differentiated toward keratinization and direct differentiation of the *stratum germinativum* into the normal type. These take place simultaneously." "The histological sequences observed in the removal of cells above the *stratum germinativum* indicate that autolysis as shown by vacuolar degeneration and heterolysis as shown by leucocytic infiltration are involved."

In repair the vacuolar degeneration separates the replacement epithelium into two strata, the upper to disappear completely, the lower to reproduce the epithelium normal for the region. The sequences are very similar to those in the rat's vagina in the di-estrus. As the metaplasia of vitamin *A* deficiency and its recovery is a cycle that probably does not occur in animals in natural habitats, it is of great interest to note that the recovery phenomena follow a familiar physiological pattern.

Perhaps the fact of greatest interest in vitamin *A* deficiency is the preservation, by the cells of the *stratum germinativum* of the replacement epithelium, of the identity of the original epithelium throughout the period of metaplasia. Wolbach and Howe found that the cells of this layer can assume their original morphology and functions without undergoing division when supplied with vitamin *A* and inferred that the nuclear chromatin remained unaffected by the deficiency. In the opinion of these authors, the cycle of vitamin *A* deficiency and recovery affords an experimental method available for the correlation of nuclear chromatin and types of cytoplasmic activities. Searching cytological studies by competent persons would probably bring to light new relations of structural detail and physiological activities.

2. *Bone and Nervous System*

Paralysis and nerve degeneration as a consequence of vitamin *A* deficiency have been affirmed and denied by a number of investigators. It has been known for many years that vitamin *A* deficiency retards growth of bone and in particular endochondral bone formation. This effect has not been regarded as different from that of inanition, however produced. Recently it has been shown by Wolbach and the author that if vitamin *A* deficiency is established at a sufficiently early age, skeletal growth becomes retarded in a unique manner a

considerable period before the rate of increase in weight is materially affected. The central nervous system and other soft tissues continue to grow at approximately their normal rate until general inanition effects appear, as shown by stationary weight or loss of weight.

The effects of this disproportionate growth of bone and central nervous system are (*a*) overcrowding of the cranial cavity, resulting in distortion of the brain, dislocation toward the *foramen magnum* and multiple herniations of the cerebrum and cerebellum into the venous sinuses of the dura at sites of arachnoidal villi; (*b*) overcrowding of the spinal cord and herniations of nerve roots into intervertebral foramina and into the bodies of vertebrae; (*c*) mechanical damage with subsequent irregular degeneration of the nerve roots, peripheral nerves and of nerve fibers in various tracts of the spinal cord and in the brain.

The reparative powers of the neurons are not impaired—at least before the effects of general inanition become apparent—as evidenced by the prompt appearance of axon regeneration phenomena on the proximal side of the mechanically damaged nerve roots adjacent to herniations.

The nervous lesions of vitamin *A* deficiency are thus wholly mechanical in origin.

Weanling rats (21 days old) on a vitamin *A* deficient diet usually exhibit signs of nerve lesions by the 54th day of age. The later the deficiency becomes established, as shown by the effect upon growth, the less frequently does the disproportionate growth reach a degree sufficient to produce compression of the central nervous system. Vitamin *A* administered at 42 days of age will prevent paralysis, although dissection may show slight degrees of relative overgrowth of the nervous system. If the vitamin deficient rat is prevented from growing by an insufficient amount of food, the nervous system preserves relatively normal relations to the skeleton and no paralysis ensues. Rate of growth and not age is the important factor in the production of the disproportionate relations. Rats maintained at a small size by insufficient amounts of a complete diet into an age period in which, in normally nurtured rats, it is not possible to produce paralysis, exhibit the disproportionate growth just as rapidly as do weanlings when placed upon a vitamin *A* deficient diet.

We have found a similar disproportionate growth of bone and central nervous system in young vitamin *A* deficient guinea pigs, evidence that this is not a species-specific phenomenon.

The prompt retardation of endochondral bone formation in vitamin *A* deficiency indicates that a specific action must be involved, but no distinctive histological appearance has been found. Osteogenesis *per se* is not inhibited because bone matrix (osteoid) formation continues late into the deficiency. .

Vitamin *C* Deficiency

1. *Ascorbic Acid*

The morphologic consequences of experimental ascorbic acid deficiency are practically restricted to supporting tissues of mesenchymal origin and may be expressed by the statement that there is failure of formation and maintenance of intercellular materials. This definite and simple characterization explains the pathology of experimental ascorbic acid deficiency in susceptible animals and in the naturally-occurring deficiency in man (scurvy). Hemorrhages, loosening of teeth, failure of wounds to heal and, in infants, extensive subperiosteal hemorrhages and separation of the epiphyses of long bones are manifestations of scurvy and all are explainable on the above simple pathologic principle.

All intercellular substances of the supporting tissues, bone, cartilage, fibrous connective tissue and dentin, have a common substructure of collagen. By collagen I wish to indicate a number of compounds of similar though not necessarily identical composition which react similarly to staining techniques. It is this protein substructure which in scurvy is either not produced or is produced in defective form.

The guinea pig has been used almost exclusively for ascorbic acid deficiency experimentation. In young guinea pigs on a diet completely deficient in ascorbic acid, microscopic evidence of retarded deposition of intercellular matrices, bone, connective tissue and dentin can be found as early as the seventh or eighth day. Finally, all deposition of intercellular material in growing structures ceases and there is slow resorption of the matrices previously laid down.

Wolbach and Howe believe that ascorbic acid is not necessary for long survival and multiplication of the cells concerned with matrix formation. The deficiency is specifically concerned with the elaboration of intercellular substance, which they regard as the product of the cells. They described in some detail the change in morphology of osteoblasts that accompanies the failure in bone matrix production. In the incisor teeth of guinea pigs, the columnar odontoblasts undergo changes in size and shape which probably represent a change in mor-

phology accompanying loss of a function. In each instance, the bone- and dentin-forming cells take on the appearances of undifferentiated connective tissue cells.

The continuously growing incisor teeth of guinea pigs provide perhaps the best material for the study of the effect of ascorbic acid deficiency upon formation of matrix. In these teeth the dentin is being continuously deposited at the formative end to compensate for the wearing away of the tooth structure at the incisal end. The rate of eruption of the lower incisal teeth of the guinea pigs is about 2 mm. per week. At the formative end of the tooth circularly arranged tall columnar cells, the odontoblasts, become differentiated from the mass of embryonic-like connective tissue, and a matrix of collagenous material appears between their basal surfaces and the epithelial cells of the enamel organ with which they were previously in contact. They move incisally at approximately the rate of tooth eruption and at the same time retreat toward the center of the pulp, leaving more and more dentin matrix behind them. This matrix becomes calcified a short distance from the ends of the odontoblasts. The intervening uncalcified tissue is called the predentin. Each odontoblast leaves in the dentin a cell process, which increases in length as the cell moves centrally. Because of their deposition of dentin in a unidirectional manner, the odontoblasts have been termed "polarized osteoblasts" by Wolbach and Howe. Since the peripheral layer of dentin is deposited first, the odontoblasts become crowded more and more closely together as they migrate incisally and centrally. As the tooth is about to emerge from the alveolar bone, the odontoblasts begin to degenerate, form dentin of an inferior quality and finally become incorporated in the calcified pulp tissues which seal off the pulp chamber. Their life cycle from differentiation at the formative end to degeneration at the incisal end occupies about five weeks.

After seven to 10 days on an ascorbic acid-free diet atrophy and disorientation of the odontoblasts and deposition of dentin of inferior quality become evident. It may contain recognizable dentinal tubules, fewer and more tortuous than those in normally formed dentin, or it may incorporate atrophic cells and resemble bone. It somewhat resembles the last product of the senescent odontoblasts in the normal incisor but occurs in greater amounts and nearer the formative end of the tooth. In complete absence of ascorbic acid for four weeks the atrophy of odontoblasts and the deposition of secondary dentin extend back to the formative end of the incisor. In the teeth of guinea pigs

given inadequate daily doses of ascorbic acid (0.3 mg.) for long periods of time the dentin at the formative end of the tooth always appears histologically normal, while slightly farther incisally dentin of inferior quality projects in long spicules toward the center of the pulp cavity.

The use of alizarin to stain vitally the dentin formed during a particular period has made it possible to measure the marked variations in rate of deposition that occur in different parts of the incisor teeth of animals on adequate diets and to compare them with the rates found in corresponding parts of the teeth of animals on restricted amounts of ascorbic acid. The rate of dentin formation in definite areas near the formative end of the tooth has been found to be uniform in animals given the same supplements of ascorbic acid. After a standard depletion period the amount of dentin deposited in this region has been shown to vary directly with the amount of ascorbic acid administered. These findings demonstrate a measurable quantitative relation between the rates of formation of an intercellular substance, the dentin, and the amount of ascorbic acid administered to guinea pigs.

The teeth are held *in situ* by the collagen fibers of the dental periosteum which are attached on one side to the cementum of the tooth and on the other side to the bone of the socket. The failure of formation, or formation of fibers of inferior quality is responsible for the loosening of teeth—another characteristic of ascorbic acid deficiency.

The repair of wounds in severe ascorbic acid deficiency is incomplete because of limited capillary formation and absence of the formation of collagen. Observations on the central portion of clots formed after excisions of muscle have been used by Wolbach to study collagen formation. In the scorbutic guinea pig fibroblasts proliferate and invade such a clot but no collagen is found. However, within a matter of hours after ascorbic acid administration new collagen appears around isolated fibroblasts deep in the clot and the process goes on to complete granulation. This is another convincing proof of the thesis that ascorbic acid is necessary for matrix formation.

Less work has been devoted to the study of bone matrix (osteoid) formation in ascorbic acid deficiency. However, there is general agreement that osteoid deposition ceases and that osteoblasts lose their morphology and take on the appearances of fibroblasts. No detailed study has been made on cartilage and tendon although it is known that both these tissues become defective in this deficiency.

The capillary bleeding so common in scurvy is the result of structural weakness, either due to defective cementing substance binding the endothelial cells together or in the collagenous supporting structures.

Changes of importance in epithelial or other tissues have not been proved to be the results of ascorbic acid deficiency.

Thiamin (Vitamin B_1) Deficiency

The striking manifestations of disturbed physiology of acute experimental thiamin deficiency (opisthotonos, extensor rigidity of legs, and other neuromuscular symptoms) are accompanied by no, or at most a few, apparently simple tissue changes. However, a chronic deficiency of this vitamin leads to nerve degeneration, a tissue change which I wish briefly to discuss. Other features of this deficiency which there will not be time to discuss but which occur in spontaneous thiamin deficiency in man (beriberi) as well as in experimental animals are cardiovascular failure accompanied by right-sided enlargement of the heart, venous congestion of the abdominal viscera, effusions into serous cavities, and edema of the extremities. Paraventricular degeneration and hemorrhage (Wernicke's disease in man) has also been reproduced in thiamin deficient pigeons and rats.

Thiamin requirements are proportional to the available carbohydrate used by the organism. Therefore, the course of the deficiency can be influenced by either of these two variables. Likewise, unless efforts are made to maintain adequate caloric intake, starvation may become a compensating factor in preventing the development of typical symptoms and lesions, or in making the deficiency more chronic in appearance. Most of the pathologic physiology and pathology of thiamin deficiency is undoubtedly explainable on the basis of the rôle of thiamin in carbohydrate metabolism. The marked involvement of the nervous system is significant when one considers that nervous tissue with a respiratory quotient of unity is totally and uniquely dependent upon oxidation of carbohydrate for its function and integrity.

Dr. Swank and the author have recently finished some experiments with thiamin deficiency in the pigeon which emphasize the variation in symptoms and lesions depending upon the severity and duration of the deficiency. The author has also duplicated most of these observations in the rat, so they are not peculiar to a species.

The acute deficiency leads to severe opisthotonos, backward roll,

and rigidity of the legs. The functional origin of the symptoms seems evident from the rapid response (few hours) to replacement therapy and from the failure to find significant nerve lesions. However, when pigeons received rations inadequate in thiamin (chronic deficiency) no opisthotonos appeared but ataxia, leg weakness, and paralysis developed, with degeneration of the peripheral nerves which began at a point most distal to the cell body and progressed centrally. Thiamin therapy, if instituted before neuron death, resulted in recovery in a time commensurate with the known rate of axon regeneration. These conclusions were based on observations made at different levels of the sciatic nerves and on the employment of suitable techniques for the study of axis cylinders and myelin sheaths. Adequate controls excluded the possibilities that these effects were due to general inanition or absence of other factors.

I hope this discussion has not been so brief and sketchy to miss its objective, which is to call to your attention some of the important changes which can occur in tissues as a result of faulty nourishment, and to suggest that experimental nutrition is a research tool which may be of great value in many studies on growth and development.

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Growth, Fourth Growth Symposium, 1942, **6**, 105-117.

THE RELATION OF SOME CHEMICAL AND PHYSICO-CHEMICAL FACTORS TO THE INITIATION OF PATHOLOGICAL PLANT GROWTH[1]

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A. INTRODUCTION

It is both a pleasure and a privilege for me to confess to you some of the results of our recent studies. This confession is made with the hope that when I have finished, wherever I have wandered technically from the straight and narrow path, you will take me gently by the hand and lead me back into the way that I should go.

Our problem is aimed at determining the factors influencing plant cells, that are ordinarily in a resting stage, to begin the enlargement and rapid cell division found in various types of atypical and pathological growth.

Historically the initiation of this work seems to spring in part from the eminent cancer pathologist Dr. James Ewing. He told me in conversation several years ago that, after the first description of crown gall, he had encouraged Erwin F. Smith to continue basic research. This was done with the hope that light would be shed upon the basic mechanism of pathological growths in both plants and animals. Smith's numerous contributions with plants in this field are well known. In turn many others, including both Dr. White and myself, have been encouraged by Smith to continue working with plants. Much of the earlier literature has been reviewed by Riker and Berge (1935).

Plant materials have some significant advantages. Among them may be listed: (*a*) large numbers, (*b*) low cost, (*c*) ease of experimental use, (*d*) large physiological variability, (*e*) easily induced epidemics, and (*f*) genetic purity of the host either by selfing or by vegetative propagation. To these should be added the possibility, so well presented on Tuesday by Dr. White, of cultivating tissue *in vitro* in a medium where every constituent is known. These advantages were recognized long ago among plants and lower forms of animal life by

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This paper is condensed from a presentation at the symposium held August 24 to 27, 1942, by the Society for the Study of Development and Growth. The photographs were made by Eugene Herrling.

Dr. Hammett and his associates, as this group well knows. The fundamental similarity in all cells is clearly expressed by Szent-Györgyi (1939):

> Biochemistry teaches us that many constituents of our body are found with equal frequency in plants and animals, fulfilling analogous functions in each. These substances of plants, just as they are, or with little alteration, fit into the machinery of our cells. Two machines, the parts of which are interchangeable, cannot be very different, and so anything we learn about the plant will lead us closer to the understanding of ourselves.

Various of my Wisconsin associates have coöperated in securing the results which I am reporting. Among these I must mention particularly Professors I. L. Baldwin (Bacteriology), B. M. Duggar (Plant Physiology), and W. H. Peterson (Biochemistry). These and others have prevented me from more frequent self-reproach so forcefully and so ungrammatically expressed, *"If I had of knew what I ought to have knew, I'd never have did what I done."*

The metabolic activity of the cell-stimulating crown gall (Figure 1) bacterium, *Phytomonas tumefaciens* (Smith and Town.) Bergey *et al.* provides the center of our discussion. This activity is most clearly defined in chemical and physico-chemical effects induced.

Thus, our position is comparable with that of investigators studying nonparasitic but pathological growths. However, the bacteria maintain some of these chemical and physico-chemical factors over a long time and in small but continuous amounts that can be achieved only with difficulty, if at all, by other experimental devices. Thus, the bacterial activity is a very useful means of examining the initiation of pathological growth.

First I wish to discuss ways in which the cell-stimulating activity of the bacteria can be reduced. I am sure you will forgive me for drawing most of my subject matter from the work done in Madison. If I were to stretch out this last paper on the program to include the work reported in a considerable literature, we should all actually and figuratively miss the bus.

B. Attenuation of Crown Gall Bacteria

Early attempts to destroy the pathogenicity of these bacteria were made with a variety of substances. At that time only negative results were secured. The pathogenicity of these bacteria is a remarkably stable character, being apparently not diminished in cultures that are

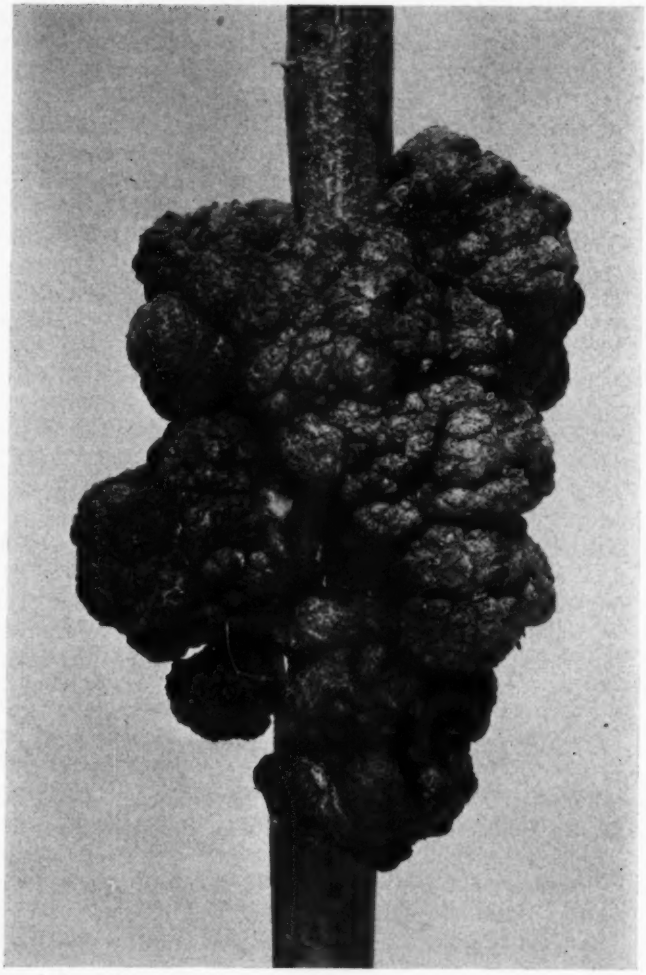

FIGURE 1

CROWN GALL ON FIELD GROWN GIANT RUSSIAN SUNFLOWER ABOUT THREE MONTHS AFTER PUNCTURE INOCULATIONS (xl.)

10, 15, and even 20 years old. However, results indicative of attenuation were secured when the cultures were placed in media containing from 0.1 to 0.3 per cent glycine—enough to reduce the amount of growth but not to inhibit it entirely (Van Lanen, Baldwin, and Riker, 1940). After 15 to 25 successive transfers in such media the bacteria were attenuated. The attenuation was more rapid in alkaline than in neutral or acid media. Although the attenuation of pathogenic cultures by means of toxic substances, heat, and antibodies is well known, so far as we are aware this is the first time attenuation has been accomplished with natural cell constituents for which chemical formulae may be written.

Similar tests were made with about 50 of the related nitrogenous compounds—all that we were able to beg, borrow, buy, or steal. Most of the stable natural amino acids were included. With the exception of dicyandiamide, which gave only partial attenuation, and the dipeptide of glycine, all the attenuating compounds were amino acids. These were: glycine, alanine, serine, alpha amino *n*-butyric acid, threonine, valine, norvaline, methionine, leucine, norleucine, isoleucine, and lysine. Only racemic mixtures of optically active amino acids were studied, except with leucine. In this case both the *l*- and *dl*-compounds were found active. Except for phenylalanine, there was direct correlation between inhibition of bacterial growth and attenuation.

Glycine and alpha amino *n*-butyric acid did not specifically inhibit either glucose or succinate dehydrogenase, aspartic acid deaminase, or aerobic respiration in the Warburg respirometer. The active amino acids, likewise, did not specifically inhibit the peptidases splitting leucylglycine, leucyldiglycine, or alanyldiglycine.

Over 1,000 attenuated cultures have been produced during these studies.

The attenuation induced by glycine is not limited to this group of bacteria but is also accomplished by a similar treatment of the legume root nodule bacteria (Longley *et al.*, 1937). Here, not only the ability to produce nodules but also the ability to fix nitrogen is reduced by the glycine attenuation.

I am particularly happy to present the results of some of the work with amino acids at North Truro because of the numerous and outstanding investigations made here by Dr. Hammett and his associates (*e.g.*, Hammett, 1942) on the physiological activity of various amino acids and related substances.

C. Restoration of Virulence

The degree of permanence achieved by the glycine attenuation depended upon the length of time the cultures were kept on the attenuating medium (Van Lanen, Baldwin, and Riker, 1940). If they were removed from the glycine medium as soon as they lost the ability to produce galls when inoculated into tomato plants and were returned to the conventional yeast infusion medium, virulence was gradually regained after 6 to 10 months. However, certain cultures that were continued on the glycine medium for 10 to 15 transfers beyond the time when attenuation was first accomplished have not regained pathogenicity after a period of five years.

This work has been based upon mechanically picked single cell cultures and has been frequently checked with single cell technique. This has avoided the possibility of merely making selections within an original mixture.

The influence of ultra-violet light was also studied in relation to attenuation (Duggar and Riker, 1940). Bacterial cultures were treated until approximately 0.1 to 0.2 of 1 per cent remained alive. When fully virulent cultures and fully attenuated cultures were thus treated, the irradiation had no perceptible effect. However, when partially attenuated cultures were treated, a change was often induced which was in the direction of restored virulence.

Another phenomenon might easily be confused with restored virulence. When a virulent culture is inoculated into a tomato stem above an attenuated culture (Figure 2), the pathological growth about the attenuated culture is approximately as large as that about the virulent culture (Locke, Riker, and Duggar, 1938). This situation is considered at greater length in connection with growth substances.

While these effects on the modification of the virulence of the cultures seem to be vaulable in relation to an understanding of their activity, there are various other means of studying these microörganisms that may also shed some light on the factors associated with the initiation of pathological growth. While it is quite impossible to cover the list of working hypotheses that have been suggested from time to time, we may advantageously consider several representative factors in the situation.

D. Physico-chemical Agencies

The temperature at which the crown gall bacteria and the tomato host plants grow varies in both cases from about 18° to 32° C. for

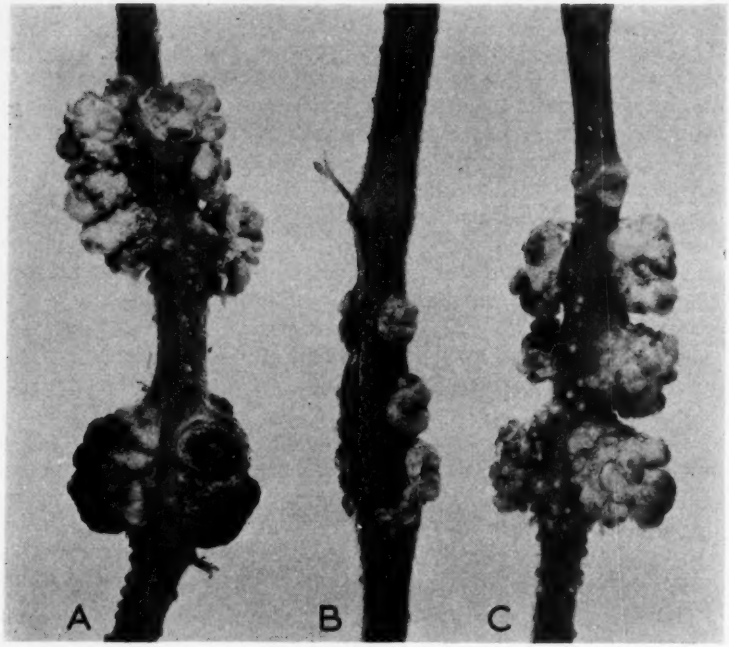

FIGURE 2

CROWN GALL ON GREENHOUSE TOMATOES ABOUT SIX WEEKS AFTER INOCULATIONS

A.—Galls resulting from a virulent culture above and an attenuated culture below. The lower galls were influenced by those above. *B*.—Galls from the attenuated culture alone. *C*.—Galls from the virulent culture alone. (xl.)

good growth. However, there appears to be a temperature between 28° and 30° C. below which crown gall inoculations develop excellent crown galls but above which no gall development takes place. Obviously, there is some critical physiological condition affected by temperature so that there is no gall development above 30°. This is probably associated with some condition of the host since crown galls develop on certain other plants, e.g., Nicotiana hybrids, at the higher temperatures (Riker, Lyneis, and Locke, 1941).

Oxidation reduction potentials have been studied with the crown gall bacteria *in vitro*. Like many others, these bacteria lower the oxidation reduction potentials of sterilized plant extracts from about +400 millivolts to about —100 millivolts, with variations depending

upon the medium and circumstances (Riker, Pinckard, and Baldwin, 1936). If something like this reduction occurs in the host tissue, as one would expect, the bacteria are obviously inducing a relatively severe condition of "oxygen hunger" in the small space they occupy between the growing cells. How important this question of "oxygen hunger" may be in traumatic injuries and subsequent tumor development in animals can be discussed much better by others present.

Osmotic pressure in culture media under the influence of bacterial metabolism was measured. While the means of studying this important phenomenon are not as exact as we might desire, it is quite apparent that the osmotic pressure increases and decreases depending upon the particular fraction of the medium being used. For example, if complex molecules are being broken down into an increased number of smaller molecules, osmotic pressure would obviously be increased. However, if a substance like sugar is being oxidized into carbon dioxide and water, osmotic pressure would be decreased. This happens very conspicuously in culture media (Riker, Lyneis, and Locke, 1941).

While the growth of the bacteria in the plant tissue is undoubtedly thus producing various physico-chemical effects, the bacteria are also manufacturing certain substances that deserve special consideration.

E. Growth Substances

The enzymatic equipment possessed by the crown gall bacteria is surprisingly complete. This organism has been able to utilize a long list of representative sources of nitrogen, including ammonium and nitrate salts (Sagen, Riker, and Baldwin, 1934). Likewise, it has been able to utilize over 30 representative sources of carbon.

Growth substances essential for the growth of crown gall bacteria are apparently synthesized by these bacteria since they are able to multiply abundantly in a synthetic medium containing only purified sucrose as a source of carbon, ammonium sulphate as a source of nitrogen, and the conventional mineral salts. Biochemical attempts to measure some of these materials have shown the presence of auxin (several reports, e.g., Locke, Riker, and Duggar, 1938), thiamin, riboflavin, pantothenic acid, and biotin (McIntire, Riker, and Peterson, 1941).

Plants bearing sizable crown galls show a variety of effects commonly associated with auxin treatment, namely, secondary thickening of stems, adventitious roots, gall formation, inhibition of axillary

buds, and prevention of the formation of abscission layers (Locke, Riker, and Duggar, 1938). However, our attempts have thus far failed to find enough heteroauxin or similarly active substance that can be detected with the Avenae coleoptile technique, to account for gall formation (Riker, Henry, and Duggar, 1941).

A phenomenon apparently associated with growth substance has already been mentioned in connection with restored virulence; viz., when a virulent culture is inoculated into a tomato stem above an attenuated culture (Figure 2), the tissue around the attenuated culture grows about as rapidly as that near the virulent culture (Locke, Riker, and Duggar, 1938). Apparently some substance passes down the stem that supplies the necessary metabolite otherwise deficient about the attenuated culture. This substance does not affect the attenuation of the bacteria because they can be reisolated and shown to be still attenuated. This procedure also shows that no virulent organisms have moved down and induced the effect by contamination of the attenuated culture.

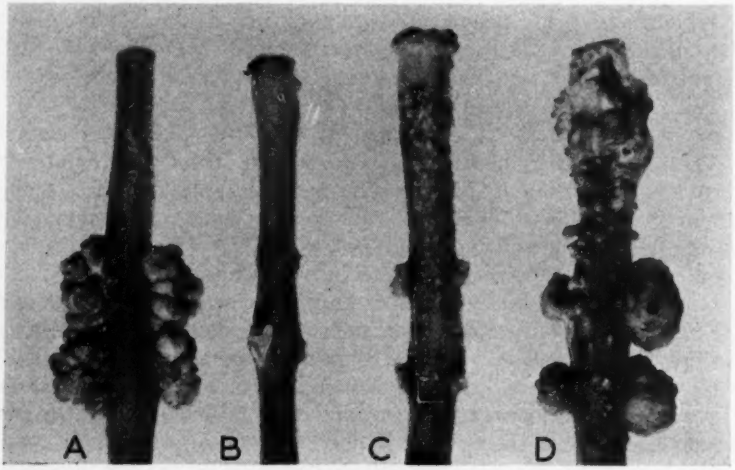

FIGURE 3

THE INFLUENCE OF CHEMICALS ON GALL DEVELOPMENT BY GREENHOUSE DECAPITATED TOMATO STEMS ABOUT FIVE WEEKS AFTER TREATMENT

A.—Lanoline alone on cut end above inoculations with a virulent culture. *B.*—Lanoline alone above inoculations with an attenuated culture. *C.*—Lanoline with 3 per cent indole-3-acetic acid above inoculations with an attenuated culture. *D.*—Lanoline with 3 per cent napthalene acetamide above inoculations with an attenuated culture. (xl.)

Comparable results may be secured if treatments with growth substances like 3 per cent indole-3-acetic acid or better still—3 per cent naphthalene acetamide are placed above inoculation with attenuated cultures (Figure 3). While one might jump to the conclusion that such a chemical is the factor missing from the region of the attenuated culture, caution is indicated because these chemicals induce considerable cell growth near the point of application. So these rapidly growing host cells may be providing the missing factor.

With regard to vitamins, galls contain two or three times as much thiamin as healthy tissue (Henry, Riker, and Duggar, 1942), and this increase appears within a week after inoculation.

While data are not yet available on other growth substances, there is interesting evidence concerning other bacterial products.

F. Metabolites of Crown Gall Bacteria

Various metabolic substances in addition to the growth substances already mentioned have been demonstrated in the crown gall bacteria or in their cultures. Dr. R. J. Anderson of Yale University has isolated a considerable number of substances, including phosphatides, phospholipides, and various derivatives (Geiger and Anderson, 1939). Earlier, the Mulford Biological Laboratories supplied him with material and, more recently, we have sent him several kilograms of bacterial cells (we called it bacterial mud from its appearance and consistency) centrifuged from several thousand gallons of culture medium. Various chemical fractions have been returned to us for biological assay. Unfortunately, our trials with these materials have been less encouraging than we had hoped because the test plants we used either responded more or less vigorously to all of these and to other chemicals applied or else failed to respond satisfactorily to any of them. With recent improvements in technique we hope to measure better the biological activity of these substances secured from the bacterial cells.

Various other metabolites occur in the medium outside of the bacteria. Considerable carbon dioxide and ammonia are produced by these organisms under suitable circumstances. Likewise, they produce a considerable quantity of material resembling gum which is doubtless responsible for the reduction of surface tension or viscosity which these bacteria induce in the medium (Berge, Riker, and Baldwin, 1936). Some of these substances appear to be very stable (Conner, Riker, and Peterson, 1937) while others are readily broken down into

glucose. One of the latter substances has recently been characterized (McIntire, Peterson, and Riker, 1942) as a polysaccharide. It appeared to be homogeneous as judged by analysis of diffusion data and by constant rotation through reprecipitation and fractionation of both the original material ($[\alpha]_D^{25} = -9^\circ$ to -10° in water, $c = 2$) and its acetylated product ($[\alpha]_D^{25} = +56^\circ$ to $+58.5^\circ$ in chloroform, $c = 1$). Hydrolysis of the polysaccharide gave only *d* (+)-glucose as indicated by specific rotation, iodometric titration, and yield of glucobenzimidazole. A shift in rotation during hydrolysis indicated a predominance of β linkages, while the rate of hydrolysis and the shape of the hydrolysis curve suggested that the inner ring structures were exclusively pyranoside. The molecular weight calculated from sedimentation velocity and diffusion constants was 3600 ± 200, which corresponds to about 22 anhydroglucose units per molecule. This material is extremely hygroscopic and may have an influence on the moisture relations and the viscosity of the immediately surrounding cells.

These bacterial metabolites and various other substances, including some carcinogenic agents, have been placed on plants and have induced "irritation" with more or less cell stimulation. In general, our results are in accord with those reported earlier (reviewed by Riker and Berge, 1935), which show that an extremely wide range of chemicals is able to induce at least some cell stimulation in plants. This stimulation seems, sometimes at least, associated with nutritional disturbances.

G. Change in Distribution of Materials in the Host

The normal distribution of food materials in plant tissue is considerably altered by the pathological growth in crown gall as indicated by proximate analyses (Nagy, Riker, and Peterson, 1938). In general, the composition of gall tissue resembled that of young plants, being high in nitrogen and low in fibrous material.

An accumulation of food materials with attendant pathological growth is very conspicuous above a wire girdle (Riker and Hildebrand, 1934), a partially cut stem, defective grafts (Riker and Keitt, 1926), or physiologically incompatible graft unions (Riker, 1928). Under these circumstances the food material is apparently unable to pass the barrier and collects, probably with a considerable accumulation of growth substances, so that very conspicuous growth occurs.

With respect to other important tests, the glutathione content of

tomato galls was greater than that of the contiguous stems but was much lower than that of the growing tips (Nagy, Riker, and Peterson, 1938). The hydrogen-ion concentrations of stems and galls of tomato tissue were approximately the same. Catalase, oxidase, and peroxidase activity, on the wet-weight basis, were 160, 130, and 120 per cent greater, respectively, in the tomato galls than in the contiguous tomato stem tissues. This is interesting in view of the "oxygen hunger" induced in plant extracts by the crown gall bacteria, as mentioned earlier. Extracts from tomato galls rapidly destroyed tyrosine, but a similar preparation from stems showed no tyrosinase activity.

While we might continue discussing chemical and physico-chemical factors associated with the initiation of pathological growth, those given will perhaps serve as examples.

H. Discussion

Before my presentation is considered in general discussion and since you obviously plan to stay to the bitter end, I seek a few more minutes to consider the significance of some of these analyses and to indicate some interrelations.

A number of these individual factors operating singly are apparently able to initiate certain small amounts of pathological growth. While some of the chemical galls may eventually approach in size those induced by the bacteria, the inciting chemicals commonly are used in much stronger concentration (e.g., 1 to 3 per cent in lanoline) than living cells provide. However, from what we know of crown gall bacteria, they operate in the host tissue over a long-continued period, manufacture substances that may be supplied to the surrounding plant cells in a low but continually maintained concentration, and provide a number of different factors that are all active at once. These factors include many that operate during normal growth.

Among these factors, as we have already seen, may be included "oxygen hunger," changes in osmotic pressure, rearranged amounts of growth substances and vitamins, "irritating" substances, and altered amounts of food materials. Any living cell, even a resting cell, that fails to react under such conditions seems very unresponsive.

While we shall continue to analyze individual factors that by their presence or absence may change normal into pathological growth, there is another possibility that deserves consideration. This is that in normal growth a number of factors may operate in suitable balance. However, in pathological growth of one kind a group of these factors

may be out of balance. Likewise, in pathological growth of another kind the balance is disturbed in some other way.

I. Summary

Plant materials are being used in investigations on various factors associated with the initiation of pathological growth. Since plants do not have, e.g., complex nervous, digestive, and circulatory systems, the physiology of their cellular growth and development is on a relatively simple basis, and pathological development can be studied with a number of important facilities. To study the factors associated with pathological plant growth, attention has been centered on crown gall, caused by the bacterium *Phytomonas tumefaciens* (Smith and Town.) Bergey *et al.*, and on comparable nonparasitic growths.

Among the important factors bearing on this problem and studied intensively are those concerned with (*a*) the loss in pathogenicity of the cell-stimulating bacteria which is induced by various amino acids; (*b*) their recovery of pathogenicity in certain media and after ultraviolet treatment; (*c*) "oxygen hunger" of the plant cells; (*d*) changes in osmotic pressure, which may cause swelling of plant cells; (*e*) the presence of unusual quantities of enzymes and of growth substances such as thiamin, riboflavin, pantothenic acid, and biotin; (*f*) bacterial metabolites, e.g., ammonia, phosphatides, phospholipides, and polysaccharides, that "irritate" plant cells; and (*g*) the presence of food materials in abnormal amounts.

When some of these and other important factors are present in unusual combinations and proportions, living cells commonly begin to enlarge and multiply. While continuing to examine individual factors, one may also well consider from the evidence available that the initiation of pathological growth may be associated with an unbalanced combination of some such important physiological factors.

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SOME PROTEIN PATTERNS IN CELLS

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Ever since the earliest investigations on the structure and properties of protoplasm, spanning more than a century, it has been realized that the complexity of the phenomena displayed by the cell require the assumption of a spatial and chemical integration of molecules into patterns such that the potentialities of the emergent organization are infinitely greater than those of the individual components. This fact is illustrated by the wide variety of terms which have been invented over the years for the complexes upon which life seems to depend; one thinks of Verworn's "biogens," Spencer's "physiological units," Altmann's "bioplasts," Darwin's "gemmules," de Vries' "pangens," Haeckel's "plastidules," Weismann's "biophores," Naegeli's micellar "idioplasm," and the more modern "gene." As the composition and properties of the proteins came to be better understood it was realized that these emergent properties depend primarily on protein molecules and aggregates arranged in specific patterns. With each advance in our knowledge of the structure and chemical properties of the proteins came new hints as to how this structural integration may be effected. Indeed, the old principle that one may learn much about the simple (protein molecules) by a study of the complex (macromolecules, fibers, etc.) is being fruitfully applied at the present time.

It will be obviously impossible in this paper to review exhaustively the recent data on the physical and chemical properties of the proteins which have a bearing on the ability of these substances to form supermolecular groupings and thereby, to exhibit certain "field" properties. Rather, attention will be drawn to a few special cases which seem significant in pointing the way along which further inquiry will be particularly profitable.

A few words may be said at the outset concerning the present status of the problem of the structure of the protein molecule. The specific construction of even the relatively simple protein molecules still defies attempts at rigorous analysis. The most serious limitation to such an analysis is that of determining with any degree of certainty and

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completeness the amino acid composition of proteins. However, the development of satisfactory methods is progressing and we may hope that a sound analytical foundation will soon be laid. It will then be possible to evaluate certain important generalizations such as that of Bergmann ('38), that the amino acids are arranged in a periodic pattern, the number of amino acids obeying stoichiometric rules. Recent analyses of collagen (Bergmann and Stein, '39) indicate that such patterns may be more complex than was originally visualized since it seems necessary to assign a dual frequency to the distribution of proline in gelatin and collagen.

While determination of the weight of protein molecules by the ultracentrifuge method, and of asymmetry by diffusion methods (Neurath, '39) yields clues as to protein architecture, the data are still only approximate. Even X-ray methods possess inherent indeterminacies which have thus far precluded definite structure analysis of proteins. The laborious survey being attempted by Bernal, Crowfoot, Fankuchen, and others will doubtless bear valuable fruits. Thus far a fairly complete analysis has been published only in the case of crystalline insulin (see Crowfoot, '41). From this it may be concluded that the Svedberg molecule exists in the crystal, and Bernal assumes the existence of submolecular units of 12 or 24 amino acid residues arranged in cubic close packing, probably bonded together through linkages of side chains such as those of cysteine or glutamic acid. This concept differs from that of Wrinch according to which the molecules exist as single polyhedra. In view of the uncertainties existing at present these suggestions must be considered only as working hypotheses.

One of the most obvious and important properties of proteins is their biological and chemical specificity. That this may depend on an underlying geometric symmetry of molecular architecture seems reasonable and attempts to unify our concept of protein structure through geometric considerations, particularly through the efforts of Wrinch ('38) and more recently of Langmuir ('39) have proven highly stimulating though not convincing to many (see Pauling and Nieman, '39 and a recent reply by Wrinch, '41). Indeed, the validity of one of the fundamental notions on which such generalizations rest, the molecular weight categories of Svedberg, is now being questioned (see Bull, '41). The present consensus appears to be that protein molecules owe their specific properties to the way in which each particular prescription of amino acids, arranged in peptide chains, is folded into

globular or asymmetric units. As alternatives to fabrics requiring integration through cyclol bonding, suggestions have been offered in which the integration depends on hydrogen bonds. Thus Huggins ('40) suggested two ways in which hydrogen-bonded chains might be folded in order to have many of the properties of the cyclol structure. The linkage of *CO* and *NH* through hydrogen bonds has been demonstrated very recently for simple molecules in monolayers by Alexander and Rideal ('41). Pauling ('40), in considering the structure of antibody proteins, offered an interesting mechanism for the folding of protein chains through the presence of proline or hydroxy proline residues at the periphery of the molecule.

In view of the uncertainty as to the structure of the protein molecules it is obvious that our knowledge of the larger aggregates in the protein ultrastructure of cells is even less sound, more speculative. The investigation of these larger aggregates has been greatly aided by the use of the electron microscope, however, and this new tool has already proven a powerful aid to the more indirect X-ray and polarization optical methods.

The formation of cellular structures through the aggregation of protein molecules is manifested chiefly in linear arrays such as fibrils or in essentially two-dimensional arrays as in membranes. It seems probable that this is a distinction chiefly of convenience, since, as will be shown below, there is no crucial evidence that protein lamellae are not constructed of a felt-work of fibrous protein strands. We may begin our discussion of cellular protein patterns, therefore, with a presentation of certain recent investigations of protein fibers.

Fiber Structure

Current concepts of the fine structure of animal fibers revolve about the now classical investigations of Astbury, according to which fibers are composed of indefinitely extended polypeptide chains integrated with each other laterally and capable of existing either in the extended beta configuration or folded upon themselves to varying extents (alpha and supercontracted states). That many animal fibers do contain such chains there can be no doubt, and the theory provides a plausible explanation of many of the physical properties of fibers. However, the X-ray data are relatively meagre and the analysis of the structure is still far from complete, particularly with reference to an interpretation of the equatorial long-spacings frequently observed in normal fibers, such as muscle (Meyer and Picken, '37; Astbury, '39).

Particularly significant in this connection is the possibility, suggested by Wrinch ('39) in connection with chromosome structure, that cellular fibrils may be composed of a longitudinal array of globular protein molecules rather than of polypeptide chains. It is clear that the formation of fibrils in cells requires as a first step the recruitment of protein molecules into linear arrays. These molecules may themselves possess a high degree of asymmetry, thus facilitating this type of preferential orientation, though this does not appear to be a necessary condition.

Recent experiments of Waugh ('41) are highly instructive in this connection. If a solution of insulin is boiled at a *pH* of 2.5, the insulin molecules aggregate to form a thixotropic gel which, when stroked on a glass slide, produces fibrils showing birefringence which is positive with respect to the direction of orientation. If the insulin solution is more dilute it will, after heating in acid solution, show strong fluxional birefringence which disappears after removal of the mechanical stress in a time depending on the concentration of the insulin solution. It is clear, therefore, that the treatment has caused the relatively small insulin molecules to join up end-to-end to form fibrous aggregates. This fibrilization is reversible, for appropriate freezing and thawing will cause reversion to the isotropic solution of low viscosity, and the cycle may be repeated as often as desired. It is stated that this treatment has little effect on the biological activity of the insulin, hence the molecules undergo little chemical alteration in the reversible process of aggregation. Waugh suggests that the heating liberates hydrophobic side chains normally not accessible to the exterior and that van der Waals attraction between these side chains furnishes the bonding energy for the fibrilization. This seems to require a polarized initial structure of the insulin molecules such that appropriate side chains are available at the ends to effect the linear aggregation. Some such polarization has been postulated to account for antibody properties by Pauling ('40). At any rate these experiments provide an example in which relatively small globular protein molecules may reversibly form fibers without loss of the properties and identity of the molecules. It is possible that reversible intracellular fiber formation may represent a similar process facilitated in the cell, perhaps, by specific enzymes.

Important information concerning the structure of fibrous molecules and of linear aggregates comes from recent investigations on the plant viruses (see Bawden, '39, Stanley, '40). Data on the weight,

size, and asymmetry factors as determined from ultracentrifuge, viscosity, diffusion, and filtration studies have now been supplemented by X-ray studies and direct observation by the electron microscope. Thus in the case of tobacco mosaic virus protein the molecule appears to be rodlet shaped with dimensions approximately 15x300 (or possibly 150)$m\mu$. The short-spacing X-ray diffractions give information about the intramolecular structure and it has been concluded by Bernal and Fankuchen ('41) that the rodlet molecule, having a thickness of 150Å and a minimum length of 1500Å, possesses a hexagonal lattice containing subunits of approximately 44x44x22Å with a possibility of a smaller subunit of 11Å cubed. These subunits are closely packed and little water penetrates between them even in dilute solutions. However, water readily penetrates between the rodlet molecules, the separation due to water being a linear function of the water content within certain limits (Bernal and Fankuchen, '41). The molecules within the tactoids remain oriented in their paracrystalline condition but the separation (due to water) between the molecules in the direction perpendicular to the fiber axis is uniform and may be of the order of hundreds of Ångstrom units. The forces responsible for this phenomenon have been discussed by Langmuir ('38). Since differential and reversible solvation is at the basis of protoplasmic structure formation the matter deserves the closest attention of biologists. Particularly the chromosomes should be suspected of employing such mechanisms in their ability to undergo great alterations in their solvation without loss of orientation and specific structure. It is desirable that similar X-ray and electron microscope studies be made of other cellular systems known to exhibit such properties.

The electron microscope studies of viruses reveal further important facts about the ability of the molecules to join up to form fibers. It had been suspected from sedimentation studies that the molecules readily form dimers. However, the electron micrographs show that the rodlet molecules may form long threads which may contain only a few molecules or may contain a great many. In such threads no discontinuities can be resolved to mark the domains of the individual molecules. This suggests that the molecules have a polarized structure facilitating a tight end-to-end bonding, a supposition for which there is some electrooptical support (Lauffer, '39). The threads may grow also by adhesion of molecules laterally. The various viruses differ considerably in this respect. Thus the electron micrographs of

some viruses show that the end-to-end, fibrous habit is more pronounced while with other viruses the lateral adhesion is greater, leading to characteristic cluster formation. Other typical differences have been found, such as the tendency for threads to end abruptly without forking or branching, as in the case of fibrin (Wolpers and Ruska, '39), or to produce much branched, reticular structures as in the case of tobacco mosaic virus (Kausche and Ruska, '39, Stanley and Anderson, '41). It seems probable that similar specific aggregation tendencies, which doubtless depend on chemical differences in the molecule, are at the bottom of specific structuration processes in protoplasm.

Concerning the physical and chemical factors responsible for the genesis of protein fibers in protoplasm little is known. Much may be learned by a consideration of the processes known to occur in a similar phenomenon, the clotting of the blood. In this case the relatively small fibrinogen molecules are converted into macromolecular fibrin strands. This process involves a complicated interplay of enzymes, kinases, antikinases, and electrolytes, the details of which are still far from clear. It is improbable that the processes of intracellular fiber formation are less complex than those of blood clotting. It is safe to predict that chemical investigation of the intracellular fibrilization mechanism, undertaken on the same large scale as that of blood clotting, would yield returns of great importance for cell physiology and perhaps for clinical medicine.

Schmidt ('24) pointed out that intracellular fiber formation is almost universally associated with the presence of one or more granules, usually of centrosomal origin (spindle and asters, cilia, sperm tails). Blood clotting is initiated by changes in the platelets and, in an important recent publication Wolpers and Ruska ('39) furnish information concerning the rôle of the granules of the blood platelets. Injury to the platelet surface causes the gradual clumping of the granules to form a structure resembling a nucleus. At the same time, due to permeability changes, the cell contour changes and processes grow out (some cells pictured in this condition resemble neurons with axon hillocks and fibers having terminal blebs). Owing to the increased permeability, prothrombin is thought to enter the cell and to react with intracellular thrombokinase, producing thrombin. Clot formation begins when the cell protruberances and vacuoles are extruded into the plasma where the thrombin reacts with fibrinogen. The first fibrin threads adhere strongly to the clumped granules which have

been denuded of protoplasm by rupture of the cells. The threads do not adhere to other cellular elements such as red cells or intact platelets but only to the granules. This explains the radial distribution of the fibers in a clot. How can one picture intracellular fibers being formed by such a process? One might suppose that regions of the protoplasm containing a kinase are separated from the rest of the protoplasm by a semi-permeable interfacial film. Some compartmentalization of this sort might be effected by the lamellar structure of the ground substance for which Monné ('40) claims to have polarization optical evidence. Breakdown of the film would cause mixing of cytoplasmic pro-enzymes with the kinase and the activated enzymes thus produced would cause certain protein molecules in the cytoplasm to be recruited into fibrous aggregates. Preferential adhesion to granules (perhaps centrosomal) would furnish the symmetry of the amphiastral figure. While this is highly speculative, it seems a reasonable supposition on which to base an experimental approach.

Considerable interest attaches to the question of the properties and identity of the protein precursor or precursors of intracellular fibrils. This subject received considerable attention in the latter part of the last century (for a brief review see Bensley, '39). Particularly as a result of the work of Wooldridge and Halliburton the conception arose that most tissue cells contain a protein called tissue fibrinogen which plays a rôle in tissues similar to that played by fibrinogen in blood clotting. The tissue coagulins of Mills ('21) belong in the same category.

Bensley ('38) believes that "plasmosin," which may be extracted from cells with 10 per cent *NaCl*, is a protein present in the ground substance and may be responsible for fiber production in protoplasm, as well as for cell elasticity and adhesiveness. The cells still retain their normal form and shape after removal of the plasmosin and extraction with alkali is required to bring into solution the proteins responsible for this shape. These proteins, called "ellipsins" by Bensley and Hoerr ('34), are similar to the "plastins" of Reinke and Rodewald. Somewhat similar experiments have been reported in a recent note by Banga and Szent-Györgyi ('40).

When water is added to a salt solution of plasmosin, fibers are precipitated out which resemble those of a blood clot morphologically and in their ability to contract. Bensley suggests that the rodlet micelles aggregate end-to-end to form the reversible protoplasmic fibrils and irreversible tonofibrils. In protoplasm the plasmosin mole-

cules are unassociated but physical forces such as protoplasmic flow might orient the particles ("phaneroplasm" of Seifriz, '31). He suggests further that the insoluble ellipsins responsible for the structure and shape of the cell may have been formed by an irreversible alteration of plasmosin. This idea is inherent in the writings of earlier investigators and in the speculations of Peters ('37).

An examination of the fine-structure of these plasmosin fibers by X-ray and polarization optical methods and by the electron microscope is highly desirable and experiments along this line are in progress in our laboratory. Such an analysis, when supplemented by a chemical investigation of the conditions which normally determine the recruitment of the rodlet protein molecules into an organized lattice, may lift much of the mystery surrounding certain cellular processes and may lead to important methods of control since cancer, for example, involves the active participation of the structural building stones of the cell.

Membranes

We know relatively little about the molecular architecture of cellular membranes. Because of their extreme thinness X-ray methods are difficult to apply. From the form birefringence of the envelope of mammalian erythrocyte stromata it has been concluded that the proteins (and lipides) occur in layers with surfaces parallel to that of the envelope (Schmitt, Bear, and Ponder, '36). This gives no information, however, concerning the molecular construction of the protein grids except that their thickness is small compared with the wave length of light. Examination by reflected light methods indicates the possible presence of discontinuities in the molecular pattern of the envelope proteins (Waugh and Schmitt, '40). Some electron micrographs of rabbit erythrocytes[1] show discontinuities which may correspond to the units of the protein lattice. However, better resolution is being sought to determine this point.

The thin protein grids in the envelope may be thought of as composed of polypeptide chains lying in planes parallel to the surface of the envelope but having random orientations within these planes. The chains may be integrated into planar fabrics resembling those pictured by Wrinch ('38). Alternatively, the protein may occur as

[1]These photographs of material prepared by Dr. W. F. Waugh and the writer were made by Dr. T. H. Anderson at the R.C.A. Company. We are indebted to Dr. Anderson and Dr. Zworykin for this kindness.

fibrous megamolecules. For example, when a dilute solution of virus protein is deposited on a thin film for examination by the electron microscope, the photograph frequently shows that the individual molecules have become integrated into a reticular network. If the envelope proteins be fibrous megamolecules they must be thinner than the virus molecules (150Å), since the entire protein in the envelope (presumably several layers) has a thickness little greater than 100Å.

The presence of fibrous protein molecules in the red cell envelope is indicated by the fact that when the stroma protein is taken into solution by solvating agents the solution shows strong fluxional birefringence. Boehm suggested the asymmetry factor may be in the thousands. Moreover, when stromata are treated with solvating agents such as perchlorate, very slender strands having astonishing length may be observed with the ultramicroscope (Furchgott, '40). It is as though the protein felt-work of the envelope had been unraveled in the form of a long strand.

Protein Patterns in Embryogenesis

It has long been suspected that morphogenetic polarizations and fields may be intimately associated with, if not actually caused by, specific patterns of oriented protein molecules. Harrison ('36) suggested that an X-ray diffraction investigation of embryonic tissues in stages of polarization or asymmetry might provide evidence for this hypothesis. The experiments were performed somewhat later (Harrison, Astbury and Ruddall, '40) but the results were entirely negative. The patterns obtained from neural plate, neural tube, ear ectoderm and notochord of Amblystoma, and from various tissues of chick embryos showed only rings corresponding to the usual backbone and side chain reflections characteristic of disoriented proteins. However, these negative results do not disprove the idea that specific molecular orientation is an important factor in morphogenesis. If the structure is highly tenuous its presence might not be revealed by X-ray methods. Thus the axis cylinder of invertebrate nerve fibers shows marked rodlet form birefringence but the orientation cannot be detected by X-ray methods (Schmitt, Bear, and Clark, '39).

The polarization optical method would be more likely to reveal the presence of such a tenuous oriented protein lattice, and some studies have been made by this method. Picken and Waddington (unpublished, quoted by Waddington '40, p. 111) state that the narrow ends of the cells lining the early blastoporal groove, which are favorable

in being free of yolk, show a weak birefringence indicating fibers running along the length of the processes, i.e., perpendicular to the outer surface of the egg. It is not stated whether this birefringence is present in the cytoplasm of the cells or is limited to the cell membranes. Hobson[2] has made a careful study of the chick embryo in polarized light. He finds that the walls of the neural folds and tube in the living state show birefringence which is positive with respect to the long axis of the cells, i.e., negative with respect to the long axis of the tube and embryo. These structures also show the metatropic shift indicating the presence of oriented lipide and protein in the cell membranes and in the cytoplasm. The presence of a slight amount of birefringence in the protoplasm leaves open the question of a cytoskeleton in the cells of the neural plate and tube. He observes little change in the birefringence of the neural folds during closure, indicating no great change in cytoplasmic orientation during this process. The cell and nuclear membranes show birefringence, indicating the presence of oriented proteins and lipides.

Lawrence, Needham, and Shen ('40) have used another method to test the hypothesis that the shapes of protein molecules are important in determining the morphological shapes of cells, and in particular that the elongation of cells to form the neural plate depends on, or is accompanied by, increase in number, size, or axial ratio of protein particles. The proteins were extracted from localized regions of the embryo and their asymmetry tested by fluxional birefringence methods. In their preliminary note it is stated that the total globulin and "myosin" fractions have properties resembling those of tobacco mosaic virus protein while a pseudoglobulin fraction has properties more nearly resembling those of ovalbumin. A limitation of this method is that, even though it be shown that large asymmetric protein molecules make their appearance at a time when a given process of embryological differentiation occurs, hence may contribute to bringing about this differentiation, it is impossible by this method to localize the protein molecules in any particular part of the cells, i.e., in the cytoplasm, in the cell surfaces, and so on. If the physical mechanism of the differentiation is to be determined such a localization must be made.

The possibility that fibers may play a rôle in determining polarity and other patterns of embryogenesis has been suggested by Needham

[2]These experimental findings are based on unpublished work of Dr. L. B. Hobson who kindly communicated them to the writer.

('36). Along the same line Waddington ('40) points out that since ordered cell movements are not characteristic of the egg as a whole nor of the cells individually, the underlying mechanism must transgress cell boundaries. He pictures "some sort of intimate structure of the protoplasm, such as a fibrisation which was not stopped by cell boundaries." Again, "Unlike the fibers of the spindle, however, those postulated to explain gastrulation would have to keep their relative arrangement on a scale larger than that of the cells." It is implied that the fibers transgress cell boundaries by means of intercellular bridges, a concept previously suggested by Moore ('30) though not insisted on by him as a demonstrated fact (see Moore and Burt, '39; Moore, '41). The evidence mentioned above indicates that if there are oriented microfibrils in the cytoplasm they must form a very tenuous system. It is difficult mechanically to see how such a system could determine cell shape and produce movements of cells. When the cells are isolated they tend to round up, giving little evidence of self-determination of shape. The latter property seems to depend on the interaction of cells in tissue masses, as in the epithelium producing the neural folds.

Another mechanism which has been suggested (Glaser, '14) as the cause of autonomous invaginations, involving a change in shape such as from the flat cuboidal or low columnar shape of ectodermal cells to the tall columnar and flask shape of prospective neural cells, depends on a swelling of the cells through water imbibition, coupled with a differential weakening of the inner and outer cell membranes (in response to organized action). If only the inner cell membranes are stretched the shape of the cells would be changed to a truncated pyramidal form producing essentially an oriented wedge. Glaser found an increase of some 200 per cent in the volume of the cells (planimeter measurements of sections). These results are not confirmed by the recent experiments of Brown, Hamburger, and Schmitt (in press) who find that the density of the cells decreases very little during elevation and folding of the neural plate, the values being essentially similar to those of the ventral ectoderm, and suggesting a water uptake of only about 5 per cent as compared with the 200 per cent expected from Glaser's data. It seems necessary, therefore, to seek a different explanation of the cause of the folding, change in shape of cells, and cell movements than that of differential uptake of water.

Important experimental data bearing on these processes has been furnished by Holtfreter ('39, *a*, *b*) in connection with his experiments

on the dynamics of the formation of entodermal and mesodermal organs. He points out that cells of common destiny do not seek each other out; rather, affinity is manifested only by cells in contact. There is negative as well as positive affinity, and cells which have banded together may part company due to the development of negative affinity. This affinity, positive or negative, is considered the cause of oriented movements of masses of cells.

Since the evidence concerning the rôle of intra- and inter-cellular fibers is negative, and since differential imbibition of water appears to be ruled out, let us see what rôle the cell surface may play in these phenomena. For a number of years it has seemed to the writer[8] that the interaction of cells in the formation of embryonic tissues might be explained in terms of reactions occurring at cell surfaces rather than within the bulk of the protoplasm. For the purpose of bringing this view into focus before this symposium it may not be inappropriate to indicate some of the evidence which favors it.

Let us consider first the importance of the limiting envelope of the cell in determining cell shape. In the case of the mammalian erythrocyte it seems clear that the characteristic biconcave shape of the cell depends on the structure of the limiting envelope for, after reversible hemolysis, the cells readily reassume the biconcave shape. Since the total thickness of the envelope proteins is 100-150Å, it would appear that these preferential mechanical properties are produced by only a relatively few layers of surface protein molecules. Because of this the determination of cell shape by the limiting envelope could occur only if the cells are essentially in osmotic equilibrium with their environment. Even relatively small osmotic imbalances would subject the protein surface film to forces sufficient to distort the normal structure of the envelopes and the cells would tend to become spherical. Moore ('41) has shown that relatively small increases of osmotic pressure in the blastocoele (ca. 0.5 atmospheres) suffices to prevent invagination. It is entirely possible, therefore, that the shape of embryonic cells may be determined by the structure of their limiting envelopes, in particular by the chemical interaction of the envelopes of adjoining cells. The possible mechanism of this chemical interaction may now be considered in more detail.

A well known example of the induction of positive affinity between

[8]The writer's interest in these problems was "evoked" by numerous stimulating discussions with his colleague, Dr. Viktor Hamburger, to whom he is indebted for guidance in embryological matters.

free cells is the formation of rouleaux by mammalian erythrocytes. In this case the intercellular bonding may be so strong that the rouleaux may be stretched like rubber tubes before they break. The adhesion seems to depend on the action of a substance or substances in the plasma. This substance may be fibrinogen since there is a good correlation between fibrinogen content and sedimentation velocity. However, other hydrophilic colloids such as acacia, gelatin, or nucleoproteins may also be effective. Abramson states that if the formation of rouleaux is due to adsorption of a protein film, this protein is present in an amount too small to alter the electrophoretic properties of the cells. It has been suggested (Fåhraeus, '29) that hydrophilic proteins desolvate the surfaces of the red cells, raising the surface energy and thus promoting adhesion.

Let us inquire more closely into the chemical mechanism responsible for this phenomenon. The electrophoretic behavior of red cells indicates a preponderance of acidic groups at the surface. It seems probable that these are the phosphoric acid groups of cephalin since this is the only acidic lipide and it is present in the envelope in concentrations higher than that of any other lipide. How may cephalin play a rôle in the aggregation of red cells?

Recent X-ray studies on cephalin may provide an answer. In dilute emulsions of cephalin there is a thick layer of water molecules between the polar interfaces of the bimolecular layers of cephalin (Palmer and Schmitt, '41). If a small amount of histone or other basic protein is added to the cephalin emulsion an insoluble histone-cephalin complex is formed (Chargaff and Ziff, '39), and X-ray studies suggest (Palmer, Schmitt and Chargaff, '41) that this is produced by the intercalation of a monolayer of histone between each bimolecular layer of cephalin at the polar interface. The strong interaction between the basic protein and the acidic cephalin causes the expulsion of the water from between the double layers and the production of an insoluble precipitate.

If red cells contain cephalin at their surfaces, might not small amounts of histone cause strong interaction between the surfaces of neighboring cells, expelling the water from between the cells and causing strong aggregation? The experiment was tried and it was found that histone does indeed cause washed rabbit red cells first to become spherical and then to aggregate in clusters, the size and shape of which depends on the mechanical agitation imposed. Linear aggregates resembling epithelia, and spherical aggregates resembling

morulae, etc., could be produced at will by proper adjustment of the conditions. This effect was obtained with concentrations of histone as low as 0.005 per cent and small clusters of two to five cells were obtained with concentrations as low as 0.001 per cent. A simple calculation shows that under these conditions the cells could have been covered by not more than a very thin, possibly monomolecular, film of protein. The adhesion of the cells was quite firm, indicating that a single molecular layer of protein between cells suffices to bond them together firmly.

Similar aggregation of red cells may be produced by a number of other reagents in low concentration, one of the most effective being thorium salts. Histone was selected in the above experiment because of the X-ray information on histone-cephalin complexes. A large variety of substances may act as "evocators" for the production of cell aggregation; some substances are effective in very low concentrations while others require higher concentrations.

In this special case the protein evocator itself, possibly as a monomolecular intercellular film, acts as an adhesive material. The adhesion of cells in renal epithelia, in tissue cultures and in capillary endothelia seems to depend on some sort of a cement substance secreted by the cells and requiring the presence of calcium ions, according to Chambers ('40) and Zweiback ('40). "Cement substances" generally probably belong in this category.

In the general case we may say that change in interaction between cells, hence in adhesion, may be brought about by any condition which alters the solvation of the cell surface. If the surface molecules be protein, these will in general be highly solvated due to presence of polar groups directed to the exterior. There will be a repulsion between cells, therefore, due to the osmotic pressure of the water molecules brought into the intercellular spaces by the presence of the gegenions. Any substance, such as calcium ions, whose affinity for water is greater than that of the protein polar groups will desolvate these groups thus favoring interaction of the groups in the surfaces of adjoining cells and producing adhesion of cell to cell. This may be the basis of the action of calcium in promoting the adhesion of blastomeres and of lithium ions in producing the abnormalities described by Hörstadius and Lindahl.

This generalization represents an extension to whole cells of the mechanism postulated by Bungenberg de Jong ('37) to explain coacervation in colloidal systems. Whole cells replace the colloidal

particles as the units which became integrated through changes in surface solvation. We may extend the speculation further and indicate how changes in surface interaction may explain changes in cell shape and cause movements of cells.

In Figure 1, *A*, are represented several flat cuboidal cells situated

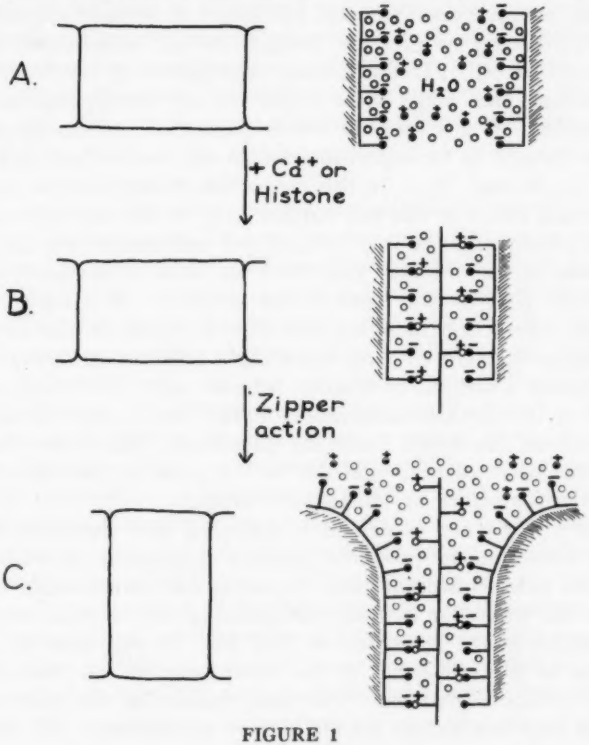

FIGURE 1

at the point of infolding of the neural plate. There is relatively little interaction between the cells since the surfaces are highly solvated. In *B* it is assumed that interaction between surfaces has been increased by the introduction of a desolvating medium (for convenience shown as a monolayer of histone, after the manner of the experiment with erythrocytes). This draws the cells tightly together, as though a zipper fastener were pulled up between the cells. As a result of this zipper action the tendency will be to increase to a maximum

the amount of surface shared in common by adjoining cells. The result is that the flat cuboidal cells will be transformed into tall columnar cells (*C*). This in turn will exert tension on neighboring cells and a movement of cells tangentially towards the infolding neural plate will result.

It may be questioned whether the forces of surface interaction are sufficiently strong to produce changes in cell shapes and to effect movements of cells. If the histone aggregation of erythrocytes is a valid analogy this would seem to provide an experimental answer to this question. At any rate, the forces postulated are exactly the same as those thought to be responsible for muscle contraction (Meyer and Mark, '30, Bernal, '38). In this connection it may also be mentioned that protein fibers in the cell surface may in the end be responsible for the phenomena. It was brought out previously that the protein component of the envelope may have the form of randomly oriented microfibrils lying in the plane of the envelope. It is easily conceivable that interaction between such protein fibrils in the surfaces of adjoining cells might not only cause tight adhesion of cells but might also produce a fibrillar continuity between cells. If certain localized regions of the surface membranes interact much more strongly than other regions this would cause the microfibrils which have interacted to become oriented perpendicular to the plane of the envelope thus giving the semblance of intercellular bridges.

Bensley ('38) and others have suggested that the proteins of the surface membrane are produced from the cytoplasmic proteins through a process akin to denaturation. It seems not unreasonable to expect that, in the life of an actively metabolizing cell, when a new protein is produced in the cytoplasm it may find its way into the limiting envelope of the cell either in the same condition or after alteration due to surface degeneration (for data concerning the penetration of proteins into monolayers see the papers of Shulman, '37; Stenhagen and Rideal, '39; and Rideal, '39). If it be discovered, through experiments such as those of Lawrence, Needham, and Shen ('40), that asymmetric protein megamolecules make their appearance at a certain stage of development, say at the neural plate or fold stage, these molecules may alter adhesion of cell to cell, change cell shape, and cause movements of cells, through their mutual interaction *at the cell surfaces* quite as well as through an intercellular fibrilization mechanism as postulated by Waddington ('40). If the reasonable point of view be adopted that there is a dynamic relationship between

the proteins (and lipides) in the surface envelope and in the cytoplasm, then the fibrisation theory of the English School does not differ in essence from the surface interaction theory here proposed. Waddington's requirement that there be some type of organizing influence which is not stopped by cell membranes but transgresses such boundaries is fulfilled in a manner more probable, and incidentally more effective, than by cytoplasmic and intercellular fibrisation. The bonding between the surfaces of adjoining cells may be quite as strong as that between the molecules within a single envelope. In this sense there is molecular, or perhaps micellar, continuity between cells in the developing embryo (the phenomenon may be of importance also in the tissues of adult forms).

In the surface interaction concept any substance which is capable of altering the interaction between the surface molecules of adjoining cells may act as an evocator. This may be effected through a primary action in which the evocator molecules become part of the surface mosaic. Thus histone would strongly evoke adhesion of cells whose surfaces contain negative charges; sterols might effect interaction because of their "sensitizing" action in coacervates (Bungenberg de Jong and Bonner, '35; Bungenberg de Jong and Saubert, '37). Or, evocation may be effected through secondary action, in which case the evocator, through effects on cellular permeability and metabolism, causes the production (or disappearance) of molecular species which, when present at the cell surface, alter intercellular interaction. This definition of primary and secondary evocator action differs somewhat from that given by Waddington ('40), in stressing the site and mode of action of the evocator rather than making the distinction chiefly one of potency of action or whether or not the substance is *the normal* evocator. The surface interaction theory emphasizes the importance of the reacting system (cell surfaces in dynamic interaction with each other and with protoplasm) rather than any one evocator or category of evocators.

A fairly good analogy to the organizer problem exists in the field of protein structure. There is the same question of pattern of subunits and the evocation of different patterns by the action of reagents. Thus ultracentrifuge data show that certain large proteins, such as thyroglobulin, though monodisperse under certain conditions, may be made polydisperse by slight alteration of the *pH* or electrolyte concentration. The several components may assume a different pattern, when reassembled by alteration of the environment, than obtained

in the original monodisperse system and the properties of the reassembled aggregate may be quite different from those of the original. Luck ('38) has discussed this phenomenon in case of the liver proteins. This component property of the proteins may be due to the protein subunits in their interaction with ions, water, etc., or it may be due to the presence of prosthetic cementing substances (Sorensen, '30; Pederson, '39). On a higher plane of organization we have the case of the megamolecules, particularly the fibrous proteins, as exemplified by the viruses (vide supra) and the collagens, elastins and reticulins (Nageotte and Guyon, '30; Nageotte, '31; Wyckoff and Corey, '36). Progressing to a still higher hierarchy we come to the microscopically visible level. There is, therefore, a gradual transition from the relatively simple organization of the small protein molecules to the complex microcosm of protoplasm. If the dynamic nature of the living, metabolizing, growing cell is kept in mind there is no reason why one should not consider the organization of cells into tissues and organs as a still higher level. The value of such a concept lies chiefly in its ability to suggest new experiments for the investigation of the forces involved in the organization of cellular patterns as in embryogenesis, by modern physical and chemical methods. No claim is made for originality in proposing this surface interaction theory, the germ of which may be found in many papers in the older literature (see Rhumbler, '23) and is implicit in the writings of many contemporary embryologists, in particular those dealing with the field concept (see Weiss, '39). It is hoped that a more explicit statement, even at the risk of criticism because of oversimplification and generalization, may be of service in suggesting the types of experiments which may prove most fruitful in approaching more closely to a solution of one of the most important biological phenomena, the ordered development of the organism.

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INTRACELLULAR PATTERNS: FACTS AND PRINCIPLES CONCERNING PATTERNS EXHIBITED IN THE MORPHOGENESIS AND REGENERATION OF CILIATE PROTOZOA*

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The present paper will set forth certain pertinent facts concerning patterns in the ciliate protozoa. Since an adequate conception of the status of the cell in Metazoa has not yet been achieved, it appears premature to decide the corollary of whether the protozoa are unicellular or non-cellular beings. We shall, however, speak of these patterns here as intracellular, meaning thereby simply that they occur in organisms not partitioned by membranes.

Studies on ciliate regeneration in particular have long shown us that these animals exhibit "in miniature" those distinctly biological problems of organic polarity, formation of a whole from a part, the coöperation of nucleus and cytoplasm, as well as many others. In another direction we have received reports from a new field concerned with the microscopic anatomy of the fibrillar systems of ciliates, instigated by the ingenious definition and demonstration of the neuromotor apparatus by Kofoid and his students, and extended by von Gelei, Klein, and many others. Today these two fields are converging, and we have the interesting task of describing not only the static structure of ciliates, but also their morphogenetic transformations in terms of a visible fiber system.

Briefly, the ciliate body may be analyzed into the following components: one or more nuclei which seem to be the chemical factories for certain essential substances, a ground cytoplasm of usually quite fluid character, and a more or less solid outer layer which bears a variety of specific differentiations such as cilia, cirri, trichocysts, bristles, myonemes, etc., all joined together by an intricate network of fibers. On the fibrillar level the organism-as-a-whole is represented by the fact that no cytoplasmic differentiation stands alone but all are interconnected as an integrated pattern.

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A suggestion which recommends itself as a guide for further research is that, among the mentioned components, the fibrillar system may be the one through which the shape of the cell and the type and pattern of differentiations of the outer layer are to be interpreted. If this suggestion prove valid, it would mean that the form of these organisms could be explained in terms of the properties of a visible fiber system, wholly extranuclear in position, doubtless formed principally of protein, yet invested with far more subtle potentialities than those ascribed by protein chemists to the simpler fiber systems which they have so far investigated. The question may be stated: Is there some as yet invisible groundwork which determines the shape and pattern of the cell following which the fiber system simply spreads over this contour and adapts itself accordingly, or is the fiber system itself the active, immediate agent in determining pattern and form? The latter alternative is given importance by the following points.

(*a*) Balamuth (1940) has recently reported concerning the heterotrich *Licnophora macfarlandi* that when the neck region separating head and base is transected elements of the fibrillar system frequently grow out beyond the edge of the body with their individual fibrils extending free into the medium to be *followed* by outgrowth of the body proper which results in their subsequent enclosure. Clearly, the fiber system here exhibits initiative in growth and is not merely invading a preexisting structure.

(*b*) In the same form Dr. Balamuth also found that only cuts which injured the fibrils of the adoral zone resulted in regeneration of that structure. A similar point has been established for hypotrichs in which regeneration follows only when cuts into the cell body transect one or more of the neuromotor fibers. Thus a direct relationship in certain forms between initiation of the regenerative process and injury specifically to the fiber system indicates the latter to be a direct causal agent.

(*c*) Transformations of the fiber system exhibit a definitely epigenetic character. Klein (1932) in particular has described many instances of the following sequence in the development of formed structures and pattern: First there is a generalized "narrow-net" system of fibers, then there appear at certain intersections of the network granular primordia of cilia and trichocysts, and finally cilia grow out from the granules, and trichocysts presumably do likewise, while certain elements of the network thicken to form the fully dif-

ferentiated fiber system. Numerous cases, involving the gradual development of organalles such as the mouth region and anal pore in relation to certain specific fiber lines have been described. Fiber systems, existing previous to the definitive differentiations which always arise in direct connection with them, thus satisfy the temporal conditions of a causal agent for such differentiations.

(*d*) Finally, it will be described shortly how in Paramecium shape of the daughter cells resulting from division is closely bound to growth of the fiber system: when multiplication of the fibers and their derivatives fails the resulting cells are incomplete and abnormal in shape.

Even were these special cases in which it is possible to separate some postulated vague and all-pervading formative agent from the specific contribution of the fiber system, the sensible procedure would be to follow the obvious generative continuity of the fiber system in favor of some unknown and invisible factor. But the facts just mentioned clearly point to the fiber system as at least the direct, if not an ultimate causal agent in the determination of the shape and pattern of ciliates.

When we consider the morphogenetic behavior of specific ciliates we find at once that they cannot all be described under one formula. Rather, studies on division and regeneration have revealed three distinct types of pattern changes which will here be illustrated by *Paramecium caudatum, Condylostoma magnum,* and two species of *Oxytricha.*

Paramecium Caudatum

We may first refer to some of the features of the finer structure of Paramecium as set forth particularly in the studies of Klein (1932) and of von Gelei (1934) whose figures will illustrate this account.

The animal presents a series of meridional rows of cilia. Each cilium terminates proximally in a basal granule. These basal bodies are connected by an interciliary fiber called the neuroneme. Adjacent to the basal granule are one or two bodies, the Nebenkörner, one of which is said to be the terminus of the neuromotor fiber, described by Rees (1922), which originates in one of the two central stations or motoria located on the anterior side of the cytopharynx.

Each cilium lies in the center of a pit (usually hexagonal in shape) in the outer surface of the cell. The hexagons are formed by meridional lines which alternate with the ciliary rows, and by cross-connectives which pull the meridians into zig-zag form and so create

the hexagons. In the center of each cross-connective lies an opening for the trichocyst. And in addition to this over-all pattern there are special differentiations including a cytopyge or anal spot, cytopharynx and cytogullet with their ciliary appendages, and two contractile vacuoles.

At the time of division the animal has the task of constructing two new patterns out of the original unit; and it accomplishes this end in the most conservative way, involving the minimum in dedifferentiation of previously existing structures. The cross-connectives of the hexagons together with portions of the gullet appear in fact to be the only parts which are broken down. The major events in division are, then, briefly as follows..

The cross-connectives of the outer polygonal system break down. There results a simple pattern of meridians running from pole to pole, surface meridians of the polygonal system alternating with the cilia-bearing neuronemes. Basal granules of the cilia now begin to multiply in a band around the center of the cell which will become the division zone. According to von Gelei this increase in granules occurs by budding of the preexisting granules.

The reason for this increase on body cilia at the time of division appears to be that the differentiations of the cell are so fixed that it cannot *remodel* each half into a whole. Instead it must *grow* the new posterior end for the anterior daughter cell, and the anterior end for the other. The new cells actually grow apart. Their combined length is always normally greater than that of the parent cell and this is due to a parallel structural increase in the ectoplasm. A proof of this relation is seen in the infrequent cases in which multiplication of the body cilia fails to occur and truncate anterior and posterior daughter cells are produced.

Richard Hertwig first described how the cytopharynx in division buds off a structure similar to itself. Bud and original structure then separate, the old pharynx going to the anterior daughter and the derived structure to the posterior. The original two contractile vacuoles undergo a decrease in size and are passed on as the posterior vacuoles of the two daughter cells. A new anterior contractile vacuole is supplied to each of the fission products by tubular invaginations of the outer pellicle, according to von Gelei, and the associated excretory pores of the contractile vacuoles are always connected, sometimes by special fibrils, to the surface fiber system.

After separation of the daughter cells, cross-connectives again ap-

pear in the surface network and the typical polygonal pattern is resumed. It is difficult to imagine how new cilia with their attendant polygons could be intercalated into this precise honeycomb network to account for subsequent growth. As a matter of fact, counting the number of cilia in the meridians of young and of old cells shows that very little structural increase does occur and that a fair part of the increase in length following division involves simply an expansion of the polygons set down during the fission period.

Three points of interest may now be emphasized in regard to this story of division in *Paramecium caudatum*:

(*a*) Except in a very minor way no dedifferentiation of formed structures occurs at all.

(*b*) All the original body cilia and trichocysts, together with their connecting fibers, are passed on unchanged to the daughter cells. In the case of the immotile caudal cilia it is demonstrable with special clarity that no replacement occurs: at no time are these cilia missing, and at no time does one see a double set. Barring the possibility that the cilia may be replaced one by one like the feathers of a bird, we have evidence here of the potential immortality not only of protozoan protoplasm but also of highly differentiated parts of the cell.

(*c*) All the new structures present in the daughter cells, excepting the new contractile vacuoles, arise in direct genetic continuity with previously existing parts, a striking case of cytoplasmic inheritance.

Since cilia and trichocysts are passed on unchanged from parent to daughter cells, it follows that these original units of ectoplasmic structure must be the same size in the youngest as well as in the oldest individuals. The question then arises: Are the cilia and trichocysts elaborated in the formation of the new ends of the fission products of a smaller size in proportion to the smaller volume of these youngest cells? Measurement has shown conclusively that caudal cilia, active cilia in different regions of the cell body, and the trichocysts are not proportionate parts formed in scale to the volume of the cell, but in all cells they are of the same size. In the ciliates we thus encounter unitary differentiations which possess what might be called an "atomistic" character. Cells of different age differ not in the size of the units of ectoplasmic structure but only in their number.

Turning now to regeneration, we find that if one of the ends of the cell is excised the pattern and shape of the fragment is not remodeled into a smaller whole. Instead the missing part is only gradually replaced by additions to the preexisting pattern. Such re-

placement may require several cell generations, during which the organism divides unequally into a normal daughter and one that is still truncate. That regeneration of the lost part is accomplished through growth increments can easily be demonstrated by starving the fragments, under which circumstance the animals may be prevented from regenerating indefinitely and manifest no other means of reconstituting the whole. Immediately that food is provided regeneration again proceeds as normally.

Paramecium caudatum stands as a unique case in regeneration of ciliates, being the only instance known in which regeneration is so delayed and may not be accomplished before the next cell division. It is likewise the only case known to me in which the regeneration of an animal can be postponed indefinitely by starvation.

When the points with regard to division in Paramecium are recalled, its behavior in regeneration follows as a natural consequence of the potentialities of this organism.

Failure of the fragment to make itself over at once into a smaller but perfect individual exhibits again the persistent tendency shown in cell division never to dedifferentiate ectoplasmic structures already formed. Such tendency may also account for the fact that paramecia so infrequently form cysts, only two or three cases having been recorded in the literature.

The slowness of regeneration of the ends of the cell can be attributed to the fact that the greatest ectoplasmic increase is in the central division zone of the cell during fission and that little increase occurs thereafter. When regeneration of anterior was compared with that of posterior ends, both Peebles (1912) and myself found that posterior ends are regenerated much more rapidly, showing the growth potential to be at least in the anterior end, a fact of some interest in regard to the assertion that these animals possess an antero-posterior gradient of metabolic activity.

It remains to explain why the cell may divide "prematurely," before regeneration has been completed. Here we touch upon the problem of the determination of size, for such fragments divide before they have attained the definitive maximum volume of the particular race in question. Clearly, attainment of a certain volume is not the factor which determines the time of division. Likewise it is possible in this organism to vary the nucleo-plasmic ratio in both directions, and such alterations neither hasten nor retard the time of division (Tartar, 1940).

I may here merely suggest a mechanism for the determination of the division time in paramecium for which the proofs are yet lacking. We have seen how growth following division involves a stretching of the polygonal network accompanied by a modicum of increase in structural units. Cells of one and one-half times the length of others have on the order of only one and two-tenths more polygons in any meridian. Might it not be then that division occurs when these polygons stretch to their limit? Since cut cells retain the same form as normal ones except for the missing part these fragments could in time attain the same extension of the ectoplasmic pattern as in normal cells and hence would divide before completion of their regeneration. In the observations on division in paramecium we saw how the polygonal and ciliary meridians are passed on from parent to daughter cells without changing in number so that with increase in size the greatest stretching would occur laterally and the structures which would experience the severest extension would be the cross-connectives of the polygonal system. But these are precisely the parts which break down during division, as before mentioned. Furthermore, an initial step in the division of *Paramecium caudatum* is the brief formation of a spindle shape by partial constriction of the two ends of the cell with a consequent bulging in the center, a process which would exaggerate the stretching of the formed structures in the central division zone of the cell. Such a possible scheme for size determination and initiation of division I mention here merely for the reason that it shows how in ciliates these important processes may eventually be related directly to the pattern properties of the fiber system.

Since in division of *Paramecium caudatum* so many structures are derived genetically from others, it is of interest to inquire whether this be a necessary relationship, i.e., whether an antecedent differentiation must be present in order that a new one be formed. In the case of the oral structures it is possible to test this relationship. By cutting the animals in such a manner that the nuclei are forced forward one can produce extremely truncate anterior end fragments from which the oral structures are entirely missing. These pieces never regenerate the mouth, and accordingly starve to death in the course of many days. From an as yet inconclusive number of more difficult operations it is indicated that even when the site of the oral structures is present they are not replaced. Thus with care one can cut out the oral structures alone, leaving the remainder of the cell intact. Again no

replacement of the feeding apparatus occurs—a further evidence of the rigid differentiation of this ciliate.

A further instance of the inability of Paramecium to remodel itself is seen in monster formation. Several years ago De Garis (1927) showed that paramecia monsters could be produced by inhibiting the final separation of two daughter cells through the action of *KCN*. Monsters, I have found, may also be produced in chain-forming races by cutting one of a pair of cells so that its enucleate end is now dragged around and supported by the uncut cell. Growth continues, but in an abnormal way, not to produce a giant individual, but rather a compound of parts of many individuals fused in a single mass. Clearly, an induced abnormality of pattern in the first stage of such a monster results in the abnormal patterning of subsequent growth.

Paramecium caudatum, and doubtless other Paramecium species of the "caudatum type," represents in sum an especially interesting case among ciliates. Dedifferentiation of formed structures remains at a minimum, and, with the possible exception of cyst formation, the differentiation does not return to a more generalized "embryonic" fiber network. Behavior in regeneration and in monster formation demonstrates for the fiber system a maximum of fixity and a minimum of remodeling. When the fiber system associated with the mouth region is entirely removed, no new mouth is formed. Hence if we were to compare this case embryologically we would liken it to a mosaic egg, such as that of the ctenophore, in which enduring defects can be produced by removal of certain portions of the pattern.

Condylostoma Magnum

Turning now to consider one of the heterotrichous ciliates, *Condylostoma magnum,* we find a circumstance which is in several ways quite different from that in Paramecium. The general points in regard to this species hold almost equally well for the related *Bursaria truncatella,* according to the excellent work of E. J. Lund (1917-18).

Condylostoma magnum is a large and elongate protozoan bearing at one end a hooded peristome fitted with a membranelle and a row of large ciliary plates. The posterior end is adhesive by reason of a sticky secretion and forms a dragging contact with the substratum which permits the animal quickly to retract itself either in defense or to point itself in quite another direction in search of food.

At the time of division the cell elongates further and obviously suffers a change in the physical state of its protoplasm, a process of

aggregation occurring which causes the animal to appear dark gray whereas it was previously more transparent and which doubtless also results in an increase in viscosity. No increase in body cilia has been observed, and certainly no loss of these structures occurs. The original peristome undergoes a partial dedifferentiation, later to reorganize itself on a smaller scale appropriate to the smaller size of the anterior daughter cell. At the same time, a new peristomial primordium appears in the middle of the cell, destined to form the oral structures of the posterior daughter. This primordium consists of two rows of cilia lying side by side, one of which will form the membranelle and the other the row of ciliary plates. Subsequent development consists in their deployment to opposite sides of the cell by a process which involves autolysis or an actual carving out of the cytoplasm between them, as will be shown shortly.

Constriction now occurs, part of the cell being remodeled to form a posterior end for one fission partner, and part to form an anterior end for the other.

The following features are to be noted in this account: (*a*) There is no change in the meridional system of body cilia. (*b*) The new oral structures arise quite separate from those preexisting. (*c*) There is a partial dedifferentiation of the old peristome together with its remodeling on a smaller scale. (*d*) Two new wholes are shaped from the original cell without growth of new ends as in Paramecium.

Since the body cilia are passed on to the daughter cells, they must be of the same size in the youngest as well as in the oldest individuals. The peristomes are, however, built in size proportional to the smaller daughter cells. Yet one may still inquire whether the unit differentiations of which they are composed are not, like the body cilia, of a single size. By comparing the largest cells with the smallest cells normally produced as well as with tiny regenerates very much smaller than the normal daughter cells, it was found that body cilia, membranelle cilia, large cilia comprising the ciliary plates, and the nuclear beads of the chain macronucleus were of the same size in the smallest as well as in the largest individuals six times their length. Here again we see how ciliates are constructed of unit differentiations of several types which vary in number but not in size as growth of the cell progresses.

A great variety of incisions may be made in the cell without resulting in regeneration; in fact it is quite extraordinary how this ciliate can repair all sorts of injuries inflicted upon it. Membranelle, ciliary

plate row, and nuclear chain may be transected, or any part of the cell be removed posterior to the peristomial field without affecting regeneration of the oral structures. But if any part or all of the peristome is removed, regeneration will be initiated. The course of regeneration parallels the development of the new peristome for the posterior daughter cell during division.

Thus, unlike Paramecium, Condylostoma shows (*a*) extensive dedifferentiation of ciliate parts already formed, together with (*b*) the potentiality of forming new oral structures not in direct continuity with the old, and (*c*) regeneration of shape by remodeling and not by growth.

This regeneration of shape is the normal antecedent to replacement of oral structures. If a small square fragment be cut from the midregion of the cell, it will soon develop an adhesive posterior end and perhaps at least partially through the action of its cilia actually pull itself out into an elongate shape. Thus a fragment that was as wide as long becomes several times longer than wide and regenerates a peristome in proportion to its smaller size.

But this goal of remodeling to the normal shape may under certain circumstances not be realized, especially when it is a matter of altering proportions in the other direction, i.e., of contracting an over-elongate part. By various means the cell may be cut so that the fragment retains an especially long and thin anterior end. These cases are of particular interest because they regenerate a peristome too small for the total mass of the fragments. Such observations have suggested the notion that the new oral structures are formed, not in proportion to the nuclear mass, nor to the volume of cytoplasm, but rather in harmony with the proportions (i.e., the width) of the actual site of differentiation.

A special circumstance permits us to study in Condylostoma the relation of the pattern of the oral differentiations to the preexisting pattern and shape of the fragment. For if the animals are so cut that only a short segment of the long nuclear chain remains, the myonemes contract and the protoplasms thereupon fold into what are often very abnormal shapes from which the animals seldom recover. In such abnormal fragments the symmetry relations are doubtlessly quite radically upset, though by reason of the nature of the material this condition is difficult to analyze. Differentiation of the rows of cilia which form the primordium for the new peristome occurs, but their original arrangement as well as the later deployment may take a variety of

bizarre shapes when it lacks the guiding normal pattern and symmetry of the fiber system. Under these circumstances the primordium, which normally appears as a straight line parallel with the meridional rows of body cilia may be looped in one direction or the other, or cast into a sinuous curve. Separation of the membranelle and ciliary plate rows then produces a very abnormal oral region incapable of gathering food, and the process of autolysis separating the two rows of cilia may in looped primordia actually gouge out a piece of the cytoplasm which then becomes separated from the cell.

These observations show that the normal symmetry and pattern of the cell is necessary, not for the actual differentiation of new parts, but for their normal arrangement.

Oxytricha

We come now to the third distinct class of pattern behavior which I shall instance by the hypotrich Oxytricha. The hypotrichs are characterized by possessing cirri, which are large fused aggregates of cilia. These structures are capable of a startling leg-like motility so that the animals appear to crawl like mice over obstructions in the culture dish. The number and position of the large ventral cirri is a constant characteristic of the species. Purely meridional units such as doral bristles vary in number with the size of the individual and are passed on directly to the daughter cells, together with new ones which are formed by budding of the basal granules of the old, according to von Gelei, just as we saw in the case of the body cilia in Paramecium.

At division double sets of new ventral cirri appear in two compact aggregates on the ventral surface. The division furrow eventually separates these fields, and the cirri later grow and deploy to their definitive locations. Only when the new cirri are adjacent to the original structures do the old cirri disappear by resorption. According to Dembowska, as a line of new cirri approach the old the latter are resorbed one by one as the procession advances. Two new sets of ventral cirri are thus formed and the old cirri are entirely dedifferentiated.

For regeneration to occur it suffices to remove in toto but one of the ventral cirri: thereupon all are replaced. From the very beginning the cirri appear as a perfect pattern. According to the figures of Reynolds (1932) whose work I am here largely following, the Anlagen field suffers lateral rotation as well as an anterior-posterior deployment of its elements. In Euplotes, another hypotrich, it has been

shown by Turner (1933) that the cirri form a continuum, being interconnected by a miniature fiber network which expands over the ventral surface. Similarly, Klein (loc. cit.) described the new peristomial field of this organism as originating in the fine, narrow-net fiber system within a mesh of the wide-net lines, the whole structure being first formed in miniature as a perfect emboitement within a unit of the larger pattern. Regeneration experiments have shown that the particular mesh or meshes of the wide-net system in which the primordium appears is not determined: a new locus is substituted for the normal site when this is removed by cutting.

Again we come to the question regarding the size of unit parts in ciliates. In the hypotrichs particularly it has been claimed that fragments regenerate proportionate parts. There seems no doubt that, as in Condylostoma, the number of meridional bristles and cirri of the adoral zone are in proportion to the volume of the cell, but in one form at least the size of the individual cirri is constant. The organism I measured is one which I have identified as *Oxytricha setigera*. Briefly, the marginal cirri, anterior cirri of the adoral zone, and large anal cirri are of constant size in the smallest as well as in the largest specimens. Thus if this organism is typical of the suborder of ciliates of which it is a member, the Hypotricha also exhibit the "atomistic" character of unit differentiations, meridional differentiations such as the marginal cirri varying in number but not in size, while the ventral cirri are constant both in number and in size in all cells of a given species.

Hence in the hypotrichs we find that the new fiber system and attendant differentiations of the ventral surface are not formed by direct growth and multiplication from the old but by a return to a finer, primitive—one might almost say, embryonic—fiber net from which subsequent differentiations arise. Correspondingly, there is no remodeling or alteration of the old differentiations and their connectives but a complete dedifferentiation of these old structures.

At this point I have completed an outline of the principal types of pattern and pattern behavior, and I trust that it has given an adequate glimpse of the nature of the material we have to deal with in the morphogenesis of ciliates. It remains for this section to mention certain generalizations which do not by any means exhaust the material but which may I hope safely be advanced.

(*a*) Ciliates show in a dramatic way that cell division is not incompatible with differentiation. The division period is in fact just

the time of greatest formation of new structures, and the two processes of morphogenesis and division run parallel in time.

(*b*) The ectoplasmic differentiations form a continuum, as emphasized by Klein, in which no part is isolated but all are integrated into a single pattern.

(*c*) There is a certain "atomistic" character to the units of differentiation, each type being of constant size after its brief development.

(*d*) The *local* character of the processes of differentiation and dedifferentiation deserves particular emphasis. Too frequently it is stated that ciliates in regeneration and division dedifferentiate and then redifferentiate. But we have seen how in Paramecium multiplication of cilia occurs almost exclusively in the specific central region during division, how in Condylostoma dedifferentiation and redifferentiation of the peristome occur without alteration in the meridians of body cilia together with the fact that in the deployment of the peristomial field there is an actual autolysis of cytoplasm occurring immediately adjacent to the differentiating ciliary plates and membranelle, and finally how in hypotrichs dissolution of the old fibrillar network and outgrowth of the new take place simultaneously. Clearly, differentiation and dedifferentiation are here not conditions which characterize the cell as a whole but are quite local events which may occur simultaneously in different regions of the cell. The specific fiber system pattern alone appears to provide an adequate scaffolding for such localization of morphogenetic processes.

I should like now to discuss the relationship of the fibrillar system to other components of the cell. As a rough beginning we may consider the other factors to be ectoplasm, endoplasm, and nucleus. Deletion experiments have been made in the attempt to segregate the individual contributions of these parts.

Long ago Verworn demonstrated that the naked nucleus alone does not regenerate the cell though it may retain its integrity for some time.

In *Condylostomum magnum* it is possible to eliminate the major portion of the endoplasm without precluding regeneration. Such a demonstration can be performed by swelling this marine ciliate in fresh water. Under the conditions of osmotic pressure the animal expands to the limit of extensibility of the outer layer. At the same time the endoplasm aggregates into a ball, usually at one end of the cell, leaving a very transparent, differentiated ectoplasm to which the nucleus is adherent surrounding an entirely pellucid interior. If now

the cell is cut so as to remove the ball of endoplasm together with the oral structures and the fragment is returned to the normal medium, regeneration of a new peristome begins at once and a perfect but transparent and very much flattened animal results. We may conclude that at least a major part of the endoplasm is not required for regeneration, and this point only serves to emphasize the localization of the morphogenetic processes in the outer layer of the cell.

While it appears that in most stages of the life history of ciliates new structures arise in genetic continuity with the old, Miss Garnjobst has shown by careful studies in Euplotes, and Lund in Bursaria, that during encystment all motor organelles are dedifferentiated. Klein claims, however, that in cysts a remnant of the narrow-net fibrillar system persists to form the basis for later ectoplasmic differentiation. The question of the dispensability of the ectoplasm wherein such fiber systems reside is difficult to decide for the reason that it has not yet been possible to obtain fragments entirely free of ectoplasm. Certain indirect evidence, however, has been gained from the ciliate Cyathodinium, endoparasitic in the colon and caecum of guinea pigs. Lucas (1932) has described how periodically the old organelles may dedifferentiate to be replaced by a vesicular primordium, lined with cilia which arises *in the endoplasm,* moves laterally to the surface of the cell, evaginates and deploys the new differentiations over the outside. From this unique case it appears that the fiber system may arise *de novo* from the endoplasm, but the case has not been studied with this question in mind. The difficult problem of whether and how fibrillar systems may originate *de novo* remains for the present without solution.

The remaining experimental possibility is to remove the nucleus, leaving ectoplasm and endoplasm alone to perform regeneration of the organism. In ciliates the circumstances are here complicated by the dimorphic character of the nuclei. Typically ciliates contain one or more micronuclei which form chromosomes and divide mitotically, together with one or more macronuclei which arise from micronuclear products but which, after once being formed, divide amitotically and never again show indications of chromosomes. The question then becomes: does morphogenesis require the presence of both or only of one or the other type of nuclei?

Schwartz (1935) has shown in Stentor that the micronuclei alone cannot support life of the cell. Conversely, in this animal, the micronuclei may be entirely eliminated without consequence to the vege-

tative processes of growth, division, and morphogenesis. In other ciliates, possessing but one micronucleus, such as *Uronychia transfuga* and *Euplotes patella,* certain races at least require both types of nuclei for the continued life of the animal. *Paramecium caudatum* exhibits a transition stage, for if the single micronucleus is removed by operation the race continues to produce normal forms but has a lower rate of multiplication owing to a slower beating of the cilia which in turn results in a decreased rate of formation of food vacuoles. The only rule that one can formulate is that ciliates vary with respect to the extent to which vegetative functions have been transferred from the probably more primitive micronucleus to that derived, special structure, the macronucleus. These results have proved disappointing, for it might have been hoped that in ciliates two distinct functions of the nucleus in the vegetative life of the cell would prove experimentally dissociable by reason of their being segregated between two types of nuclei. Instead, we find, as instanced by *Paramecium caudatum,* that elimination of the micronucleus affects the cell only in a very general, not easily defined manner.

Observations on all types of protozoa have demonstrated the necessity of the macronucleus for regeneration of fragments. Enucleate pieces may live for many days, are capable of energy metabolism as shown by their continued activity, yet all anabolic processes cease and formation of new differentiations never begins. Neither also are existing differentiations ever resorbed. Even when the enucleate fragments are prepared after formation of replacing structures has begun, the differentiation comes at once to a stand-still, demonstrating that whatever the contribution of the nucleus may be it is not stored in the cytoplasm and must be supplied continually throughout the progress of regeneration.

In ciliates it is possible to study the spatial relation between the nucleus and the site of the differentiation of new structures; and Condylostoma is especially favorable for this problem. The cell may be split so that the nucleus resides in one cytoplasmic mass which is united by only a thin bridge of protoplasm with the enucleate portion. Yet normal regeneration of the peristome occurs in the enucleate portion though the nucleus is far removed and must exert its action over a thin strand of cytoplasm.

In other ciliates, such as Frontonia, the nucleus is of rather indefinite location and, as in Paramecium, is separated from the outer

layer of the cell by a fluid endoplasm which may remain in constant circulation.

These facts strongly support the conception that the function of the nucleus is to supply certain essential substances for the support and possibly the indirect control of morphogenetic events in the fibrillar system, while the direct effort in the localization and formation of pattern resides in the fiber system itself, a conclusion first advanced by Klein on the basis of the obvious involvement of the fiber system in pattern formation.

In this final section I wish to point out certain provocative parallels between the morphogenetic behavior of ciliates and cases encountered in experimental embryology and related fields.

(*a*) It is well known that starving flatworms continually rebuild themselves into smaller but still perfect wholes as they consume the substance of their bodies. Dembowska (1938) has recently shown that when the hypotrich *Stylonychia mytilus* is placed under conditions of starvation the organism decreases in size and repeatedly reorganizes itself on a smaller scale.

(*b*) The fact that the ciliate cell may be cut into a great number of fragments each totipotent in the formation of a new individual is, of course, a case analogous to the familiar isolation of blastomeres from the cleaving egg and in fact attracted the attention of such embryologists as Lillie and Morgan who sought the minimal size fragment which would regenerate the whole. These were of the order of one fiftieth part of the whole, yet regarding smaller pieces it is impossible to tell whether totipotency was lost or merely that deleterious physiological conditions appeared with diminution in size of the fragments. The observation that the unit differentiations of ciliates are of an "atomistic" character may now logically define the end-point for such fragmentation, since a time would come when the piece was so small that the constituent units presented such disproportion to the feeding organelles constructed out of them that the latter could not be formed in any complete way at all.

(*c*) Just as one may derive several organisms out of a single individual, so one may produce a single individual from two, as eggs may be fused to form a giant embryo. This phenomenon has been closely approached in the large, blue-green *Stentor coeruleus*. I found that if two cells are cut and then immediately pressed together before a secure precipitation membrane could be formed around the naked

protoplasm, the two will then fuse as one. When the original structure of the two components has not been violently altered by the operation a double individual is formed. But if the cells are more closely fused the major components of each reorganize to form a single giant Stentor.

Since it has now been demonstrated that grafting in ciliates is possible, we can expect that in the future it will be tested whether a specific part of the fiber system, say the posterior region of convergence of the fibrils, is reorganized according to its position in the larger whole.

(*d*) In *Stentor coeruleus* one can also perform an experiment which resembles in some respects the reconstitution of sponges and certain coelenterates from dissociated cells. Thus if an individual is cut nearly in two the parts rather marvelously fuse together again. This operation may be repeated for an indefinite number of times in all directions of the cell, and, by perhaps the twentieth time, the whole organization is deranged, the antero-posterior rows of cilia being broken up into a mosaic of patches running in all directions. The fact that a small bridge of protoplasm is maintained between the parts produced by any cut has merely the practical significance of keeping the parts to be fused always adjacent to one another. In fact, completely isolated bits will fuse with the original mass if separated only briefly, or the needle may pass entirely through the mass, division occurring in front of the moving glass and fusion behind it. Such masses regenerate a perfect Stentor which thus arises through fusion not of isolated cells but of a number of dissociated parts of a single cell. In this demonstration we see how multitudinous isolated parts of the fiber system can reorganize into a whole.

(*e*) Polarity problems of such interest in Metazoa find their counterpart in the Protozoa. Such forms as *Bursaria truncatella* and Condylostoma have antero-posterior, and dorso-ventral axes, as well as right side and left. In both forms heteromorphic or bipolar forms have been described. Lund (loc. cit.) found a certain incidence of heteromorphic forms in Bursaria. These were individuals with two anterior ends pointed in opposite directions. All axes except the dorso-ventral were reversed in the posterior half of the cell, i.e., the body cilia in that region beat in a direction opposite to those at the other end of the cell, and the new peristome showed the normal direction of asymmetry.

The bipolar forms which I have observed in Condylostoma cultures (Tartar, 1938) showed a conspicuous difference from those described

by Lund. A new peristome was formed on the ventral surface of the posterior end of the cell, but the left-right symmetry was retained with the result that the new differentiation was a *mirror image* of the old. Reversal of the antero-posterior axis at one end of the cell was such as to result in a perfectly formed oral structure, yet the body cilia continued to coördinate with the original anterior end and with the result that this end dragged the other around behind it. Even if the original anterior end peristome was cut off the new posterior differentiations did not attain dominance over the body cilia and the animals invariably died from the simple fact that they were always running *away* from their food! It is significant, however that the new, heteromorphic oral structures showed such dominance that a peristome was not regenerated at the original anterior end. These observations demonstrate that in a single cell there can occur such dissociation of axes that some are changed while others are not.

Neither Lund nor I have been able to produce these reversals in polarity at will, but when this becomes possible we may possess material in the ciliates for a study of polarity and axis determination in the single cell.

(*f*) Double formations are often encountered in the materials of experimental embryology and regeneration of Metazoa. Such also is the case in ciliates. If the anterior end of a Condylostoma be split the two parts are so active that they seldom fuse together again. Under these circumstances a new peristome is regenerated for each portion of the anterior bifurcation and each bears the normal asymmetry, i.e., they are not mirror images. Similarly, if the posterior end of the animal is split and the parts remain separate, two posterior differentiations form, each dragging against the substratum in the normal manner. Thus the organization of the unicellular animal permits a doubling of some of its parts while retaining unity in others.

Frequently, too, in regeneration of Condylostoma there appear double peristomial fields in direct contiguity and these were always *mirror images* of one another, suggesting the principle of limb reduplication in salamanders.

(*g*) Finally, the differentiation of a cell in relation to its position among other cells, so familiar to experimental embryology, is also exhibited by certain protozoa as was revealed by the interesting work of Summers (1938) on the colonial vorticellid, Zoöthamnium. The colony, arising by attachment and subsequent divisions of a migrant ciliospore, develops into a structure of exquisite symmetry, bearing

branches which alternate in regular manner. A well-defined apical cell by division generates additional side branches. Normally the terminal branch cell is the only zooid on a branch which continues to divide. The first branch cell develops into a migrant ciliospore which may separate from the stalk and begin a new colony. Apical cell, terminal cell, branch cell, and basal branch cell are thus the four morphologically or physiologically defined cell types. That their determination is not fixed was shown by altering the hierarchy of the colony.

If the apical cell is removed, the terminal cell of the highest branch becomes the new apical cell, giving rise to new branches. When both the apical cell and the terminal branch cell of the topmost branch are removed, the subterminal branch cell—which normally would not have divided further—may become the new apical cell, or more commonly, the terminal cell of the penultimate branch is so transformed. Similarly, when the colony is transected centrally in the region where the ciliospores differentiate, a presumptive ciliospore becomes the new apical cell. The fate of an individual cell thus depends upon its position in the colony. That the integration is not nutritional is suggested by the fact that each zooid is a feeding individual. Eventually the coördination may be referred to the fiber system which has been described for vorticellid ciliates.

The analysis of these cases with respect to events on the level of the fiber system remains for future investigations, yet from the foregoing account it is clear that in the underlying changes of the fibrillar system should be sought the basis for these transformations.

My reason for discussing these parallel cases involving formation of one individual from two, of several individuals from one, partial duplication of parts, mirror imaging of double parts, reversals in symmetry and others is that they indicate that the fiber networks of ciliates constitute a system capable of the same types of morphogenetic transformation demonstrated in the embryology and regeneration of Metazoa. The analogies are of course as yet only suggestive in the vaguest way. It is difficult to imagine how any similar system could pervade an organism with discontinuous cell boundaries, yet even the simple fact that many embryos are ciliated may give us hope that an unbridgeable gap does not exist and that studies in the one field may illuminate those of the other, possibly at least by providing a suggestive model for cytoplasmic morphogenetic activities.

It must frankly be admitted that the field of protozoan morphogenesis has not yet yielded special contributions of general applicabil-

ity, yet I believe that in the future its material may be found uniquely suited for studies in the two directions I have already indicated. On the one hand, the ciliates present us with a living fiber system having morphogenetic capacities and providing a link with the studies on inanimate protein fibers. We may expect that future study with the electron microscope will reveal to us a great deal more than we now know concerning the intimate structure and behavior of these fibers. And in another direction the ciliates may represent unique organisms in which there is magnified and brought into visible relief something not too remote from the type of cytoarchitecture required as an explanatory principle in interpreting the data of experimental embryology.

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DEVELOPMENTAL PATTERNS IN SIMPLE SLIME MOLDS

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A. INTRODUCTION

To the majority of biologists the term slime mold immediately suggests a large, multinucleate, protoplasmic mass which is subsequently capable of producing relatively complex fruiting bodies of intricate and beautiful pattern. It is a natural response. First, this is the picture presented in botanical texts; and second, such plasmodia and fruiting structures are regularly encountered in the field. Also included among the slime molds, or Mycetozoa, however, are certain other forms which are seldom mentioned in texts, and which, because of the delicate character of their fructifications, are rarely if ever seen in the field. Yet, these forms are among the most widespread representatives of the group and are in many respects the most interesting. I refer to the pseudoplasmodium-forming Acrasieae.

The Acrasieae are characterized particularly by their unique fruiting phase in which great numbers of individual myxamoebae enter communal organizations, termed pseudoplasmodia, and subsequently build coöperatively unit fruiting structures, or sorocarps, of inherently determined pattern. They differ from the true Myxomycetes, or Myxogastres in certain fundamental respects. First, there is no flagellate stage comparable to the swarm cells of the Myxogastres; second, true plasmodia are not formed since no fusion of cells occurs at any time; and finally, fruit formation is accomplished by the integration and differentiation of large numbers of individual cells, whereas in the true Myxomycetes it is effected by the progressive cleavage of large multinucleate plasmodia.

Considering these differences, Jahn (12), Pascher (17), Cook (6), and others have questioned whether the two types belong together, while van Tieghem (30), Harper (8), and others have maintained that the two groups are closely allied. Not only is there a lack of

*The Northern Regional Research Laboratory is one of four regional laboratories authorized by Congress in the Agricultural Adjustment Act of 1938 for the purpose of conducting research to develop new uses and outlets for agricultural commodities. These laboratories are administered by the Bureau of Agricultural Chemistry and Engineering of the U. S. Department of Agriculture.

agreement concerning the phyletic relationships of different slime molds, but likewise the proper taxonomic assignment of the whole group is a disputed question. They have been variously considered as primitive plants (8, 6, 11) and as colonial protozoa (5, 32, 7, 1). While a reasonable defense of either position appears possible, it is, I believe, sufficient to say that the Acrasieae are very simple organisms which apparently belong somewhere near the divergence of the plant and animal kingdoms.

Because of the sharp separation of their vegetative and fruiting phases, members of this group, and particularly species of *Dictyostelium* and *Polysphondylium,* afford unparalleled material for the study of certain development processes. To an extent that is seldom approached in other organisms, the phenomena of growth and of form development here occur as distinct and successive stages. Growth in these forms consists in an increase in the mass and number of individuals (8) and is regularly concluded prior to the initiation of any developmental processes. Conversely, all growth ceases when the fruiting stage begins and the myxamoebae align themselves toward definite centers of aggregation in the initial phase of pseudoplasmodium formation. Beyond this point there is neither an increase in the size nor in the number of individuals and all further development consists entirely of the integration and subsequent differentiation of myxamoebae already present. This is important for it enables one to study, per se, growth phenomena and morphogenetic processes of development. To realize fully the significance of this fact and the potentialities for certain types of experimentation that it affords, one has but to contrast this condition with that prevailing in the higher plants. Fructification in the latter forms may, and commonly does, represent a terminal phase of development, but in any case it is invariably and inextricably allied with growth phenomena. An additional characteristic commending these slime molds for experimental study is the fact that the whole developmental cycle is normally completed within 2½ to 3 days.

Growth in the Dictyosteliaceae is a comparatively simple matter. Once a myxamoeba has ingested and digested food, increased in mass, and divided into two daughter cells, the maximum complexity of the vegetative phase has been attained. Beyond this point growth is a purely repetitive process and under favorable conditions continues until the available food supply has been exhausted. At no time nor in any species do feeding myxamoebae show any consistent orienta-

tion with respect to one another nor any degree of organization among themselves. Organization, on the other hand, is the very essence of the fruiting phase. Myxamoebae of wholly different spore origin and of initially equal potentialities come together in enormous numbers, integrate themselves into a coöperative functioning whole, and subsequently differentiate along specific lines within prescribed limits to produce a single fructification. During the entire process, progressive stages in development are initiated and completed without the establishment of protoplasmic bridges or intercellular connections or associations of any kind except surface contact. Or, stated differently, a unit fructification is developed by a colony, or society, of individual cells where the only force making possible such development is the intercellular organization which pervades the mass of myxamoebae prior to and during a period of cell differentiation.

In relating how initially unorganized and similar independent cells enter societal organizations and subsequently contribute to the production of unit fruiting bodies of specific pattern, I shall draw from the developmental history of six different slime molds. However, I shall center my discussion around the single species *Dictyostelium discoideum* Raper (20), first, because I am particularly familiar with this organism, and second, because in certain of its developmental stages it so singularly exemplifies patterns of cell collectives.

B. Materials

Discussion will be limited to members of the Dictyosteliaceae which have been studied in culture under a wide variety of conditions (24, 26). Based upon the form and pattern of their mature fructification (Figure 1), these species may be characterized in the following manner:

1. *Dictyostelium discoideum* Raper (1935): Sorocarps consisting of erect tapering stalks, or sorophores, arising from and surrounded at their bases by flattened plates, or basal disks, and bearing, at their apices, white to pale yellow spore masses, or sori.
2. *Dictyostelium mucoroides* Brefeld (1869): Sorocarps consisting of long flexuous sorophores, occasionally branched, bearing milk-white sori.
3. *Dictyostelium purpureum* Olive (1901): Sorocarps as in *D. mucoroides*, except sorophores unbranched and sori deep purple.
4. *Dictyostelium minutum* Raper (In press): Sorocarps diminutive, characterized by short, thin, commonly branched sorophores and white to colorless sori.
5. *Polysphondylium violaceum* Brefeld (1884): Sorocarps consisting

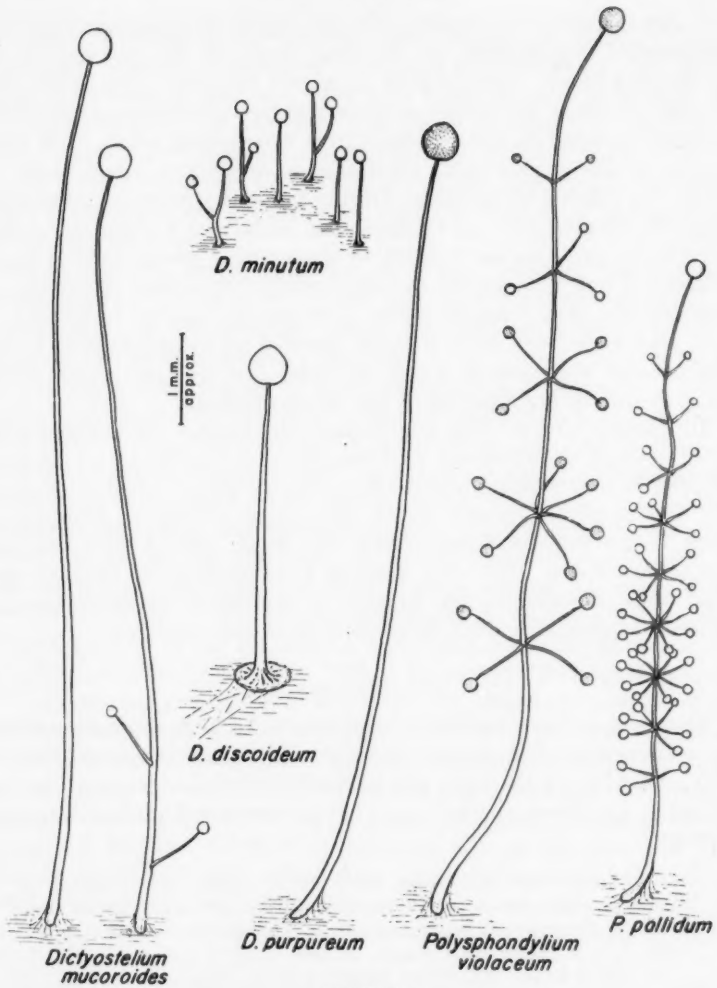

FIGURE 1

SEMI-DIAGRAMMATIC FIGURES OF THE MATURE FRUCTIFICATIONS, OR SOROCARPS, OF THE SLIME MOLDS INCLUDED IN THIS STUDY

of long axial sorophores bearing whorls of lateral branches, the terminal stem and each side branch bearing a violet colored sorus.

6. *Polysphondylium pallidum* Olive (1901): Sorocarps smaller and more compact than the preceding; sori white to colorless.

Of many bacteria tested as nutriment for these slime molds, three species have been found especially useful, namely: *Escherichia coli* (Migula) Castellani and Chalmers, *Bacillus megatherium* de Bary, and *Serratia marcescens* Bizio (*Bacillus prodigiosus* Flügge). The first species has been most used and has, in effect, served as the standard source of nutriment; the second has been employed in studying the feeding habits of myxamoebae; and the latter has been used to secure differential coloring of pseudoplasmodia (25, 26).

For detailed information concerning the laboratory cultivation of these slime molds, the reader is referred to the writer's previous publications in this field.

C. Vegetative Phase

Spore germination in *Dictyostelium discoideum* and in other species of *Dictyostelium* and *Polysphondylium* is accomplished by a swelling of the spore content followed by a longitudinal splitting of the spore case and the emergence of the protoplast as an amoeboid cell, or myxamoeba. The myxamoeba thus released feeds by the ingestion and digestion of bacterial cells, increases in size, and subsequently divides. The daughter cells in turn feed, grow, and multiply, and the vegetative stage thus initiated normally continues until all of the available bacteria (22) have been consumed. The feeding myxamoebae are of irregular and constantly changing form, move at random, and show no consistent orientation.

The general form and dimensions of the feeding myxamoebae of a single species remain essentially the same irrespective of the bacterial species with which it is associated or the medium upon which it is cultivated. In *Dictyostelium discoideum* these are of irregular and variable form and size. Commonly, however, they are more or less triangular, being somewhat broadened at the anterior end. Zones of ectoplasm and endoplasm are clearly evident and irregular extensions of the former layer commonly occur in the form of pointed pseudopodia. The single nucleus can generally be identified near the center of the body even in unstained individuals. A contractile vacuole occupies a position at the posterior end of the cell and while two or more such vacuoles may occasionally be seen, they regularly merge before being discharged.

While I have not had the opportunity to compare them histologically, morphologically the myxamoebae of other species appear strikingly similar to those of *D. discoideum*, both in their general appear-

ance and in the variety of forms that they assume. Under constant conditions the myxamoebae of species of *Polysphondylium* appear slightly more irregular than do those of *D. discoideum,* but this difference, even if established as persistent and real, is not of sufficient magnitude to be of diagnostic value. Slight differences in the average dimensions of myxamoebae of different species and strains exist, the myxamoebae of *D. discoideum,* for example, averaging slightly larger than those of other species. However, differences such as the above are difficult to measure with certainty and are, in fact, of questionable significance because of the inconstant shape of individual bodies and the marked size variation existing within any given culture. Finally, not only are the myxamoebae of different species of *Dictyostelium* and *Polysphondylium* strikingly similar but they are likewise indistinguishable from certain small uninucleate, non-flagellate, and non-cyst-forming amoebae of the soil. Knowing that a particular species has been planted, one may seem to detect differences, either real or imaginary; but facing an unknown organism of this type, I know of no morphological criteria that makes possible its identification in the amoeboid state.

The similar appearance of the myxamoebae of different species of the Dictyosteliaceae during their vegetative stage is clearly illustrated in the Camera Lucida drawings of Figure 2. I realize fully that the impracticality of identifying slime molds by their feeding myxamoebae is actually no different from that of identifying fungi by their vegetative mycelia. Nevertheless, I emphasize their similarities here as a means of showing that the distinctive patterns which subsequently appear in their fructifications arise not from obvious differences between the building units of the several species but from inherent differences governing the manner in which these units organize and differentiate to effect fructification.

As we have already noted, the vegetative stage normally continues until the available bacteria have been consumed, and throughout the entire period the population remains unorganized and reveals no consistent orientation among its constituent members. The myxamoebae move at random, feed, grow, and divide—each behaving independently of every other individual except as adjustments are necessary in their competition for food. Thus we can truthfully say that the vegetative phase of the Dictyosteliaceae is without apparent pattern save for the constantly changing and non-specific form of individual amoeboid cells.

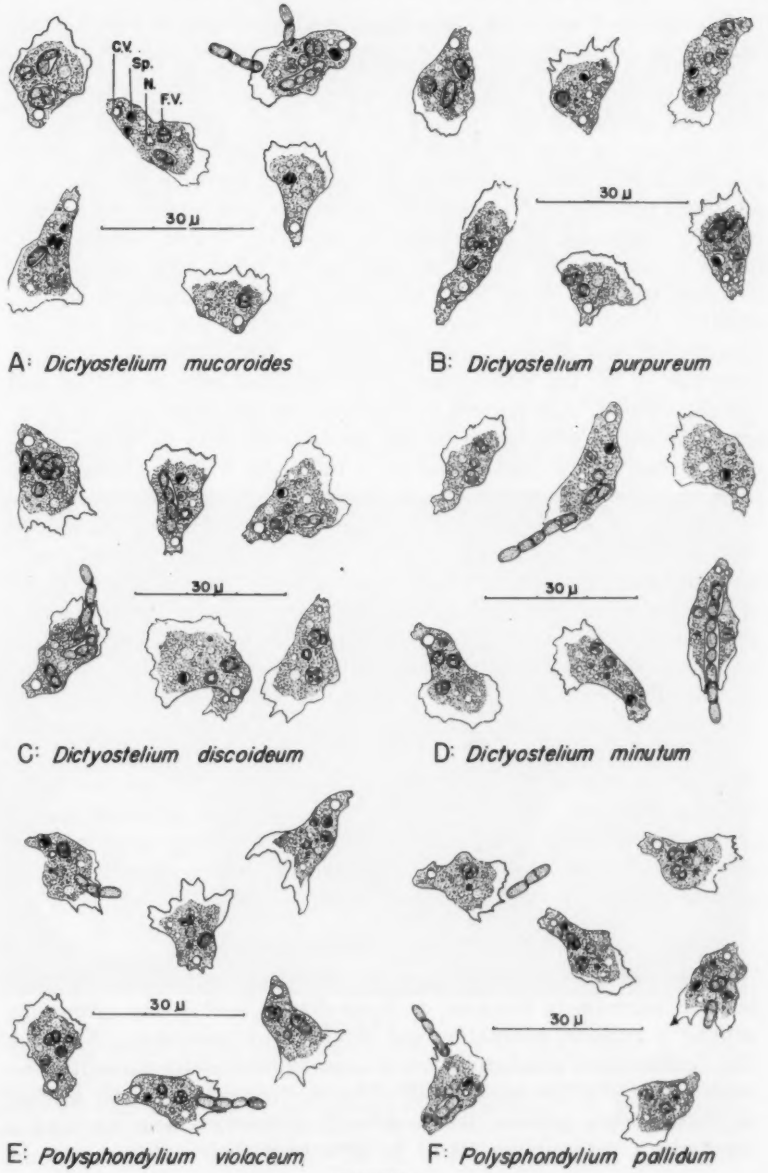

FIGURE 2

CAMERA LUCIDA DRAWINGS OF FEEDING MYXAMOEBAE OF EACH OF THE SIX SPECIES SHOWN IN FIGURE 1

Cultures grown in association with *Bacillus megatherium* upon hay-infusion agar at 22° C.±. All figures drawn to a constant scale as shown. *F.V.*, food vacuole; *N.*, nucleus; *Sp.*, undigested bacterial spore; *C.V.*, contractile vacuole.

D. Fruiting Phase

In marked contrast to the patternless condition of the vegetative stage, a succession of striking developmental patterns begin to appear with the advent of the fruiting phase. In the early stages of the fruiting process these patterns are quite similar in different species and are, in fact, generally indistinguishable. However, as fructification advances they become increasingly dissimilar and finally reach their ultimate expression of individuality in the formation of mature fruiting structures of the consistent and specific forms that characterize different species. What is, I believe, of particular interest to this audience is the fact that these specific patterns do not arise from the aggregation and differentiation of either a definite number of myxamoebae, or by the differentiation of individual cells in a fixed and specific manner. The pattern of the sorocarp represents a group characteristic and is determined by the whole community of closely coördinated individuals functioning as a unit. As I shall subsequently note, an individual myxamoeba in contributing to stalk formation may assume one of a variety of forms (p. 67) depending upon the dimensions of the stalk and the position the cell occupies in it; in any case, however, if proper culture conditions prevail, it contributes to the establishment of a sorocarp of the particular pattern characteristic of the species. The remarkable thing about this process is not the complexity of the sorocarps constructed, for they are, after all, quite simple. The remarkable thing is the manner in which fructification is accomplished; one might almost say, the fact that it takes place at all.

Just why fruiting structures of markedly different form should emerge from pseudoplasmodia of similar pattern is, as yet, not clear. We can only (*a*) observe that the myxamoebae of different species possess the capacity to organize and differentiate along consistent and specific lines and (*b*) relate the manner in which these capacities become evident in the developing fructifications.

E. Pseudoplasmodium Formation

At the outset of the fruiting phase the myxamoebae cease feeding, become increasingly elongate, or limax-shaped, and in any given area assume a uniform orientation and direction of movement. Although the fundamental characteristics of pseudoplasmodium formation remain essentially the same in all cultures irrespective of the number of myxamoebae present, this commonly influences their size and is regularly a determining factor in governing their outward pattern.

Hence it will be necessary to consider the process as it occurs under different cultural conditions. While almost any species of the group would serve equally well for tracing the process of aggregation, I shall continue with *D. discoideum* and later consider such minor variations in behavior as are characteristic of other species.

In relatively thin cultures of *Dictyostelium discoideum* growing in association with *Escherichia coli* upon hay infusion or 0.1 per cent peptone agar (25), pseudoplasmodia normally develop in the following manner. At the close of the vegetative stage the myxamoebae occur as a fairly uniform but interrupted layer of cells as shown in Figure 3, *A* and A_1. Generally, the first indication of aggregation consists in the appearance of small concentrations of myxamoebae at more or less scattered intervals. Once established these clusters of myxamoebae (Figure 3, *B*) exert some influence of unknown nature upon individuals outward from them so that they become oriented in the direction of these centers and flow toward them. As the myx-

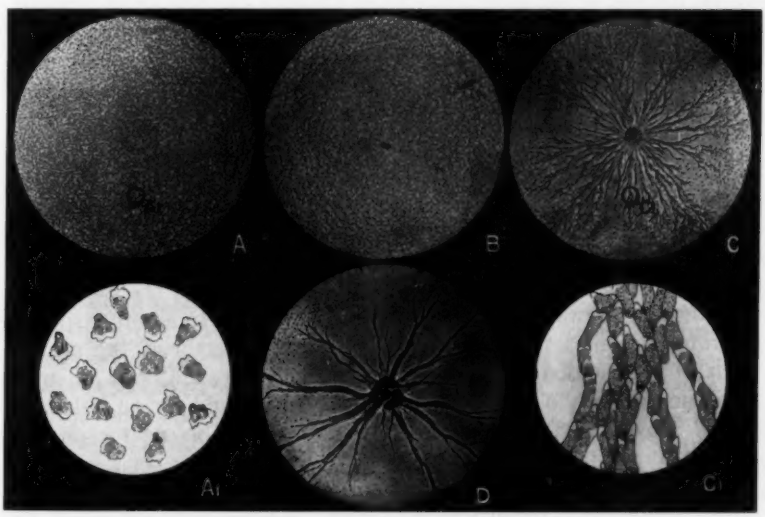

FIGURE 3

PROGRESSIVE STAGES IN PSEUDOPLASMODIUM FORMATION IN *Dictyostelium discoideum*
A, close of vegetative stage; A_1, enlarged view of small portion of colony, myxamoebae unoriented; *B*, early stage in aggregation, pseudoplasmodial center prominent; *C* and *D*, progressive stages in orientation and aggregation of myxamoebae; C_1, detail of aggregating myxamoebae showing their elongate form and consistent orientation. *A*, *B*, *C*, and *D*, x 9; A_1 and C_1, x 300 approx.

amoebae move centripetally toward the center of aggregation they collect into definite converging streams, and the whole pseudoplasmodium typically assumes a radiate, wheel-like pattern (Figure 3, *C*). Microscopically the streams are seen to consist of much elongated and similarly oriented myxamoebae all moving in the same direction and at approximately uniform speed. For the most part, the streams, once established, are continuous and the myxamoebae lie in close apposition one to another (Figure 3, C_1). However, interruptions in the smaller stream regularly appear and disappear without in any way altering the appearance or behavior of the pseudoplasmodium as a whole. The myxamoebae move as independent units and in response to stimuli apparently communicated through the culture medium. The formation of a pseudoplasmodium does not rest upon continued and uninterrupted contact of its constituent myxamoebae, but by the like stimulation and response of the multitude of individuals comprising it. As the myxamoebae continue their movement toward the aggregation center, the size of the streams increase whereas the number of streams is progressively reduced. In contrast to the earlier stage, streams are now regularly continuous and when breaks occur they normally persist, the severed fractions subsequently functioning as separate organizations. Under optimum conditions, however, the convergence of myxamoebae is typically complete except in pseudoplasmodia of very irregular pattern or very large dimensions, and aggregation continues without interruption until the entire number of individuals entering the organization have collected into a compact, cylindrical mass at the central point of aggregation. Typically this assumes the form of an upright peg and becomes progressively raised into the air as the myxamoebae outward from it continue to converge. In all other species stalk formation is initiated at this time and directly at the central point of aggregation. In *D. discoideum,* however, the body of myxamoebae normally assumes a horizontal position and for an indefinite period moves as a unit across the agar surface before forming an upright sorocarp.

In cultures of this type the first formed pseudoplasmodia are regularly radiate and symmetrical in pattern. Subsequently other pseudoplasmodia develop at their peripheries and these are generally of variable form since they originate in irregular areas of previously unaggregated myxamoebae. In any case, however, they consist of definite converging streams of myxamoebae and collect into typical migrating pseudoplasmodia. Under the above conditions, developing

pseudoplasmodia commonly measure 5 to 10 mm. in diameter, and, judged by the size of the migrating bodies they subsequently produce, contain from 50,000 to 75,000 myxamoebae (p. 56).

In like cultures, and frequently in the same plates, pseudoplasmodia of exceptionally large dimensions may develop along with aggregates of intermediate size such as we have just described; and in every culture, particularly after the initial pseudoplasmodia have developed, numerous small organizations arise.

In very rich cultures, myxamoebae are extremely abundant at the close of the vegetative stage and are present, not in a single interrupted layer as described above, but as a continuous film, commonly several layers deep. Pseudoplasmodia do not form by the inflowing of extended streams but rather by the blocking off of myxamoebae into irregular masses from which subsequently arise typical migrating pseudoplasmodia. In very rich cultures developing pseudoplasmodia commonly measure only 2 to 4 mm. in diameter but are relatively thick and frequently produce migrating bodies containing up to 150,000 or more myxamoebae. The basic phenomenon of aggregation of independent cells into collective units is unchanged, however, and apparent differences in the process here and in thin cultures is attributable solely to a difference in the quantity of myxamoebae involved in the two cases.

In very thin cultures the picture is likewise different. Either relatively few pseudoplasmodia may develop, which are characterized by very long and commonly irregular streams of myxamoebae, or a large number of small organizations may arise. In the former case the resulting migrating bodies are no larger, and are usually smaller, than those formed in thin cultures. In the latter case they are unusually small, comprising only a small fraction of the number of myxamoebae contained in the larger aggregations.

Although pseudoplasmodium formation normally follows the course described above, frequently the appearance of definite centers of orientation is not the first evidence of aggregation. Occasionally, in fact, such centers do not appear until the process of orientation and ensuing aggregation is relatively well advanced. In *D. discoideum*, and especially in *D. purpureum* and certain strains of *D. mucoroides*, I have commonly encountered cultures where great expanses of myxamoebae will be similarly oriented but devoid of any obvious centers of aggregation. It is of particular interest that when centers appear in such cultures they frequently consist, not of points toward which

the myxamoebae flow directly, but vortices into which they flow at a tangent. In his comprehensive study of pseudoplasmodium formation in *D. mucoroides,* Arndt (1) encountered and illustrated as atypical a similar type of aggregation. A field in which numerous pseudoplasmodia of this pattern are developing is shown in Figure 4,

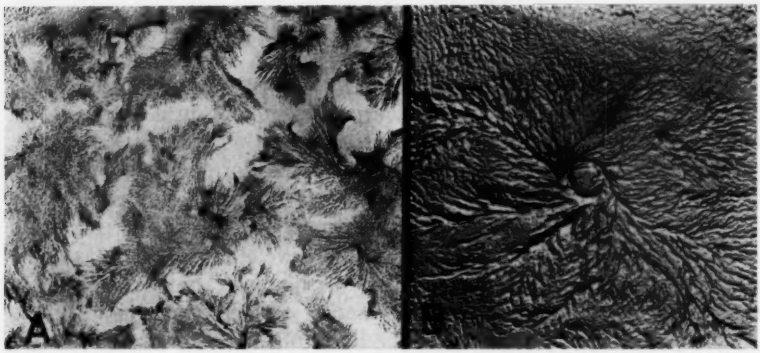

FIGURE 4

DEVELOPING PSEUDOPLASMODIA OF ABNORMAL PATTERN IN *Dictyostelium purpureum*
A, field showing a number of pseudoplasmodia with vortical centers, x 4. *B,* enlarged view of a single pseudoplasmodium of this type, x 10.

A, while in Figure 4, *B* is shown, considerably enlarged, a single pseudoplasmodium. The orientation of such aggregates may be either clockwise, or counter-clockwise, as illustrated.

Following the stage shown in Figure 4, *B* the myxamoebae normally continue their influx along the lines already established. In this process the myxamoebae eventually become more concentrated at one point than elsewhere and further convergence is toward this center but still within the same vortical pattern. In rare instances the myxamoebae aggregating toward such a vortex may join forces with an adjacent pseudoplasmodium leaving the crater-like center isolated. Upon complete aggregation, the myxamoebae typically collect into a migrating pseudoplasmodium which, in its pattern and behavior, is indistinguishable from one arising out of a radiate pseudoplasmodium. Conditions favoring this pattern of development have not been determined; but in my experience it usually appears when the agar surface is relatively wet, and when the temperature of incubation is a little below the optimum for the species.

Whereas the aggregation of myxamoebae follows essentially the

same course in each of the six species, nevertheless certain differences in behavior may be observed. Most conspicuous is the persistently small dimensions of the pseudoplasmodium of *D. minutum* and the marked tendency of these pseudoplasmodia in very thin cultures not to show conspicuous centers of convergence until aggregation is relatively far advanced (27). Upon hay infusion agar, pseudoplasmodia of this species usually arise by the blocking out of masses of myxamoebae somewhat in the manner described for *D. discoideum* in richer cultures (22). This may occur in any species when its pseudoplasmodia are exposed to an unfavorable environment, but in the present instance it takes place under optimum cultural conditions. In *D. mucoroides* and *D. purpureum*, pseudoplasmodia generally are indistinguishable from those of *D. discoideum* until just prior to the beginning of sorocarp development.

F. Organization and Behavior of Pseudoplasmodia

Under favorable environmental conditions, all of the myxamoebae entering a pseudoplasmodium characteristically collect into a compact cylindrical mass and, in due course, build a single fruiting structure. Since the pseudoplasmodium accomplishes a unit function it may, in a true sense, be regarded as a unit. It is, however, a collective or communal unit rather than an organic one, for it can at any time be resolved into its constituent myxamoebae and these will immediately resume independent conduct. As I have already noted, the pseudoplasmodium is a unit of variable rather than fixed proportions. Under optimum culture conditions, pseudoplasmodia of different dimensions regularly arise in the same culture plate, and commonly pseudoplasmodia already in process of formation break up into a number of smaller organizations. Experimentally, this type of behavior can be produced at will by altering the cultural environment, e.g., (*a*) by increasing the temperature of incubation, (*b*) by exposing cultures to dry air, and (*c*) by exposing cultures to bright light (25).

Comparable results can likewise be obtained by performing certain manual operations upon the pseudoplasmodia themselves. For example, if the central portion of a pseudoplasmodium such as illustrated in Figure 3, *D* is removed the myxamoebae comprising each stream will collect into a separate and typical migrating body. What is of particular interest is the fact that, irrespective of its origin or its dimensions, each migrating pseudoplasmodium behaves in like manner and characteristically forms a sorocarp of like and specific pattern.

Its size may vary enormously, depending upon the number of myxamoebae contributing to its formation, but its pattern remains the same whether it is formed by an entire pseudoplasmodium as originally developed or only a small fraction of the same. At the very outset the pseudoplasmodium arises by the like response of great numbers of individual cells and its progressive development rests upon the coördinated and orderly movement and behavior of these same myxamoebae.

That individual myxamoebae retain their capacity for individual response and movement throughout the periods of pseudoplasmodium formation and migration is best illustrated by the relation to bacteria of pseudoplasmodia and their constituent myxamoebae. Under normal circumstances, the myxamoebae entering the pseudoplasmodium characteristically proceed without interruption to the formation of a mature fruiting structure, and this may take place alike in the presence or in the absence of added bacteria (25). However, if in the presence of bacteria, a pseudoplasmodium is completely broken up so that its constituent myxamoebae become separated, the individuals thus dissociated will immediately return to the vegetative stage and the fruiting stage will not be renewed until the available bacteria have been consumed. If a pseudoplasmodium is only partially disorganized, only the dissociated myxamoebae will resume vegetative growth, while the remainder of the pseudoplasmodium, or intact portions of the same, will continue to develop normally. In the total absence of bacteria, similarly dissociated myxamoebae re-aggregate into minute, but typical, pseudoplasmodia and give rise to diminutive sorocarps, whereas intact fragments each produce, without interruption, a sorocarp of a size consistent with its mass. It would seem, therefore, that so long as the myxamoebae comprising a pseudoplasmodium are able to maintain certain critical relations one to another, the organization and, therefore, the unity of the pseudoplasmodium persists. When these relations can no longer be maintained, individual myxamoebae again behave as wholly independent organisms, revegetating or re-aggregating as the case may be; but the pseudoplasmodium, as a unit, ceases to exist.

While I have related observations made upon *Dictyostelium discoideum,* the behavior of pseudoplasmodia and of individual myxamoebae in each of the other species is wholly comparable. As early as 1880, van Tieghem reported that when a pseudoplasmodium of *Dictyostelium* was crushed, its myxamoebae resumed vegetative

growth. Olive (16) subsequently repeated this experiment but concluded that there was no return to the vegetative phase. He observed the formation of multiple fruiting bodies but believed this to represent only a reorganization of myxamoebae already present. Working with *D. mucoroides* Oehler in 1922 re-affirmed van Tieghem's observations, and since that time Arndt (1) has considered at greater length the relation to bacteria of the fruiting myxamoebae of this species.

While it is virtually impossible to distinguish between the larger species until the aggregation of myxamoebae is practically complete, marked differences in development begin to appear as the process of aggregation draws to a close. In all species of the Dictyosteliaceae, the myxamoebae collect into an upright peg-like mass (24) and in all species except *D. discoideum* sorocarp formation is begun without delay and is invariably initiated at the site of aggregation (Figure 6). In *D. discoideum,* as we have previously noted, a unique condition prevails. Soon after its formation the initially upright body of myxamoebae assumes a horizontal orientation and for an indefinite period prior to sorocarp formation, migrates as a unit across the agar surface.

We have already considered the origin of migrating pseudoplasmodia and have observed that while these characteristically arise by the complete aggregation of the myxamoebae comprising a single developing pseudoplasmodium, they may arise by the fragmentation of a pseudoplasmodium already in process of formation. In any case, however, they are typically of the same general pattern which may be roughly described as cartridge-shaped. The main body is more or less elongate and of fairly even diameter, whereas the anterior end is regularly somewhat pointed (Figure 5).

Structurally, the migrating pseudoplasmodium is very simple and consists of a mass of undifferentiated myxamoebae surrounded by a non-cellular sheath or envelope apparently secreted by the whole mass. Contrary to earlier reports (20, 25) that the constituent myxamoebae are regularly elongate and oriented with their long axes in the direction of movement, Bonner (2) has recently demonstrated by means of serial sections that these vary not only in form, but in their orientation in different parts of the pseudoplasmodial body. Irrespective of their position, however, when the myxamoebae are dissociated in water or on a moist agar surface, they immediately round up, draw apart, and subsequently behave as separate and unrelated amoeboids.

Of all the characteristics of migrating pseudoplasmodia, size is the most variable and is at the same time apparently the least important.

To dramatize the extreme size variation which is regularly encountered, Camera Lucida drawings of pseudoplasmodia ranging from the largest to the smallest seen in a recent series of cultures are presented in Figure 5. To emphasize even more this difference among the

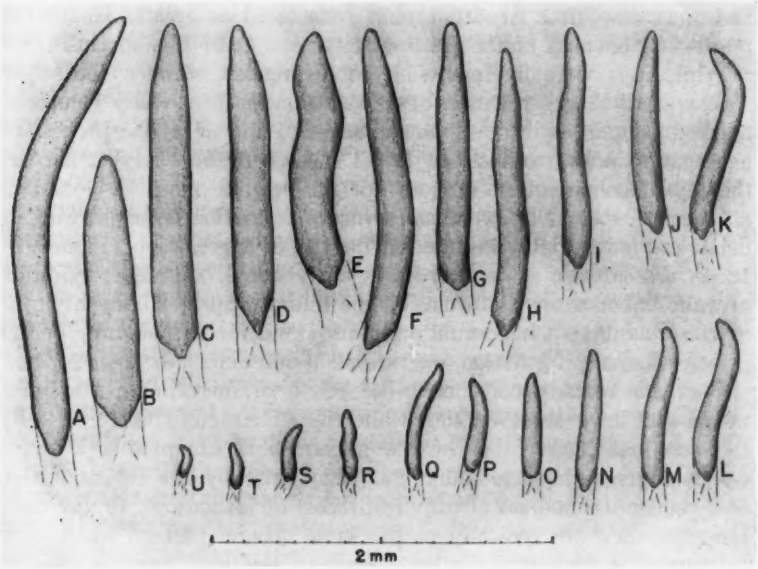

FIGURE 5

CAMERA LUCIDA DRAWINGS OF MIGRATING PSEUDOPLASMODIA OF *Dictyostelium discoideum* SHOWING THEIR ESSENTIALLY CONSTANT PATTERN AND EXTREME VARIATION IN SIZE

The estimated number of myxamoebae contained in each of the pseudoplasmodia is as follows:

A:	189,000	*F*:	131,000	*K*:	24,900	*P*:	8,700
B:	174,000	*G*:	77,600	*L*:	21,200	*Q*:	7,900
C:	165.000	*H*:	62,500	*M*:	18,700	*R*:	2,200
D:	149,000	*I*:	48,500	*N*:	15,500	*S*:	1,850
E:	136,000	*J*:	44,500	*O*:	10,300	*T*:	1,450
						U:	770

bodies shown, I have calculated the approximate number of myxamoebae contained in each pseudoplasmodium and these estimates are given in the figure legend. Subject to the inaccuracies of the method employed,[1] it is found that the largest body contains approximately

[1]Making allowances for their cone shaped apices and for their receding posterior ends, pseudoplasmodia were considered as cylinders and their volumes determined. The average

250 times the number of myxamoebae included in the smallest pseudoplasmodium.

Pseudoplasmodia are known to migrate in response to light, increased temperature, and certain chemotactic stimuli (25). In the absence of such directive forces, migration is at random. The pseudoplasmodium is strongly polarized, and whether migration is at random or in response to definite stimuli, the pointed apical end is always to the fore. When the direction of migration is shifted, the apical portion first turns in the new direction and the main body follows the lead thus established. As previously reported (25), there is apparently located near the apex of the body a center of stimulus reception, termed the "receptive center," and a center responsible for directing the conduct of the whole body, termed the "directive center." Possibly the two are identical. Certainly they are closely allied, for the two functions are intimately related. That migration is controlled from near the apex of the body can be easily demonstrated. By removing the apical portion of a pseudoplasmodium, the further migration of the body can be terminated. On the other hand, by subsequently grafting to this body a foreign apical tip the migration of the whole can be re-initiated.

In its response to stimuli and its unitary movement, and more particularly in its capacity to produce a single fruiting structure of constant and specific pattern, the migrating pseudoplasmodium of *Dictyostelium discoideum* unmistakably bears the hallmarks of a unit. But its unity, even at this stage, is in no sense fixed or stable. Under the most favorable circumstances, a pseudoplasmodium will commonly split dichotomously, giving rise to two daughter pseudoplasmodia. Conversely, two pseudoplasmodia of separate origins may merge longitudinally, giving rise to a single migrating body. In either case the resulting pseudoplasmodia are wholly typical and subsequently give rise to normal sorocarps.

The unsettled unity of the migrating pseudoplasmodium may be experimentally demonstrated in a number of ways. (*a*) If the apical portion of such a body is removed and in its place is carefully sub-

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volume of single myxamoebae was next determined by securing the average diameter of myxamoebae after the pseudoplasmodium had been dissociated and the individuals had rounded up. The approximate number of individuals contained in each pseudoplasmodium was then obtained. The method is admittedly inaccurate for the length of the calculated cylinder was in each case estimated. Furthermore, the size of individual myxamoebae varies widely. Nevertheless, it is believed to give a reasonable approximation of the number of individual cells in bodies of different size.

stituted the apical portion of another pseudoplasmodium of comparable size, the two fractions will merge and subsequently produce a single normal sorocarp. And by grafting red apical portions (obtained from cultures with *Serratia marcescens*) on colorless bodies (obtained from *Escherichia coli*) it can be demonstrated that the apical portion regularly forms the stalk, whereas the main body forms the sorus and basal disk (25, Figure 9). (*b*) The body of a pseudoplasmodium can be completely disrupted by grafting to its sides the apices of additional pseudoplasmodia. The myxamoebae immediately posterior to such added apices become controlled by them and subsequently follow their direction as they draw apart (25, Figure 7). (*c*) If a migrating body is divided transversely into two or more sections, and these are separated one from another, the apical fraction will normally continue to migrate for a period before forming a sorocarp, whereas each of the other fractions will immediately form a fruiting structure of characteristic pattern and of a size determined by its mass.

G. Sorocarp Formation

Let us now consider the manner in which the multitude of individual cells comprising the pseudoplasmodium differentiate to effect fructification. Basically the process[2] is the same in each species, but the patterns developed in sorocarp formation differ markedly. As in discussing earlier phases of development, I shall first review the process of sorocarp formation in *D. discoideum* and then compare with it the fruiting of other species.

Following an indefinite period of migration (Figure 6, *A*, 3-6), and just preceding sorocarp formation, the pseudoplasmodium ceases forward movement as a unit. Its constituent myxamoebae, however, continue to crowd forward and collect into a rounded apiculate mass at the position of its anterior end (Figure 6, *A*, 7-8). During these changes, the anterior portion of the pseudoplasmodium shifts its position, surmounts the mass, and in subsequent development governs the formation of the sorocarp and contributes directly to the building of the sorophore (25). The basal part of the sorophore is first differentiated near the center of the mass. Somewhat above the level of the substratum a hyaline tube is developed and within this the cells

[2]For this process Bonner (2) has recently suggested the very appropriate term "culmination." Not only is this term commendable from a descriptive point of view, but it is consistent with the terminology regularly employed for other developmental stages, viz., germination, multiplication, aggregation, and migration.

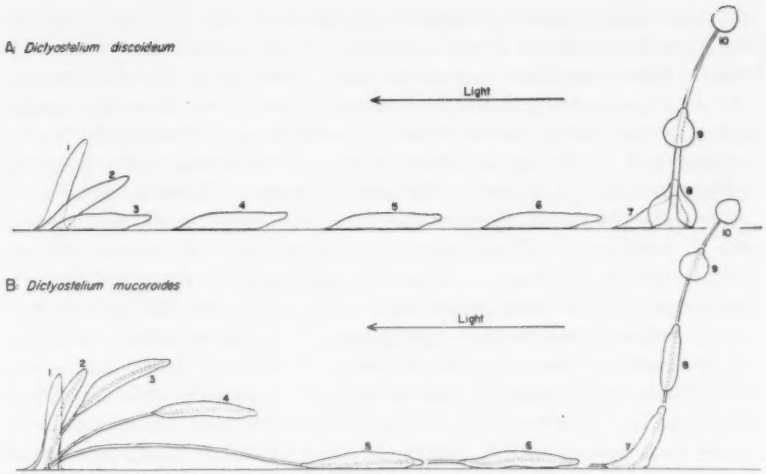

FIGURE 6

COMPARATIVE LIGHT RESPONSE IN *Dictyostelium discoideum* AND *Dictyostelium mucoroides*
(Explained in text. x 15, approx.)

become vacuolated and compacted together. It is not known whether this membrane, or sorophore sheath (8), is formed by the myxamoebae contained within it or by those surrounding it, for the myxamoebae on either side appear equally undifferentiated. In either case, however, it is the product of multiple cells and represents a function of the whole mass for its diameter is regularly proportional to the size of the entire body. As the myxamoebae within it begin to differentiate, the sheath is extended upward and downward. In the meantime a second membrane appears some distance above the base of the sorophore and is gradually extended outward in all directions to the periphery of the mass. The cells enclosed beneath this membrane become vacuolated in like manner as the cells of the stalk and form the basal disk which anchors the young sorocarp to the substratum. The upward elongation of the sorophore continues by the extension of the slime sheath and by the differentiation within it of myxamoebae into stalk cells. In the area of sorophore formation progressive stages in the differentiation and vacuolation of myxamoebae into parenchyma-like stalk cells occur from the apex downward. As the sorophore elongates the mass of myxamoebae ascends or is carried up by the developing sorophore. At about the time the mass, termed by Harper

(8) the sorogen, becomes raised above the basal disk, the myxamoebae at its periphery and in contact with the air begin to differentiate into spores. Spore maturation is progressive from the periphery inward. The two processes of cellular differentiation now proceed simultaneously and the formation of the sorocarp is completed by the transformation of all the myxamoebae either into supportive cells, forming the sorophore, or into spores, forming the sorus (Figure 6, *A*-10).

Sorocarps of quite different pattern are formed in *D. mucoroides* and *D. purpureum* (Figure 1) by modifications of the above process. Neither species possesses a migrating pseudoplasmodium, and in each case sorocarp formation begins at the site of aggregation and is commonly initiated before the aggregation of myxamoebae is complete. As in *D. discoideum,* the first evidence of sorocarp formation is the appearance of a centrally placed hyaline tube and the vacuolation within it of a column of cells. In neither species does the second membrane (see above) appear, hence no basal disk is formed. Sorophore elongation follows the course described above, involving (*a*) the upward extension of the sorophore sheath delimiting a column of myxamoebae, and (*b*) the vacuolation and fitting together of these units to form a shaft which supports the rising sorogenic mass. While it is not regularly true except in very thin and well-illuminated cultures, characteristically the sorocarps of both species may be said to be borne perpendicular to the substratum (Figure 1, *A*). In the formation of these structures, spore maturation, in contrast to *D. discoideum,* is normally not initiated until sometime after the mass of myxamoebae has lost contact with the substratum. Consequently, in sorocarps of comparable size, the sorophores of these species are generally much longer than those of *D. discoideum.*

Marked similarities are apparent between the responses of the young sorocarps of these slime molds and the migrating pseudoplasmodia of *D. discoideum.* Of these the most striking is the response to light, which is diagrammatically illustrated in Figure 6. Whereas the pseudoplasmodium of *D. discoideum* migrates toward the light for an indefinite period and then builds an erect sorocarp, the young sorocarps of *D. mucoroides* and *D. purpureum* commonly build their sorophores toward the light for a like period before the initiation of spore formation. In side-illuminated cultures, sorocarps start to develop perpendicular to the substratum but soon shift their orientation toward the direction of the light source (Figure 6, *B,* 2-3). The sorophore, which is relatively of much smaller diameter than that of *D. discoideum,* is

unable to support the mass of myxamoebae in this position and the body falls to the agar surface. Subsequently it continues to build in the direction of illumination and in contact with the substratum. Shortly before the beginning of spore maturation, however, its orientation is again shifted and further development is essentially perpendicular to the culture medium (Figure 6, *B*, 7-10). In cultures incubated in darkness the general behavior of the young sorocarp is the same except that it is not built in any consistent direction unless governed by some other stimulus, e.g., temperature.

Young sorocarps of *D. purpureum* and *D. mucoroides*, as illustrated in Figure 6, *B*, 5-6, are structurally comparable to the migrating pseudoplasmodia of *D. discoideum* except for the presence of the developing sorophore, for spore formation has not yet begun and the main body of myxamoebae remains undifferentiated. The behavior of their constituent myxamoebae is likewise comparable. If blocs of myxamoebae are separated from the young sorogen, each such mass will form a separate fruiting structure; if the myxamoebae are dissociated in the presence of bacteria they will individually return to the vegetative stage; if they are similarly dissociated in the absence of bacteria, they will re-aggregate into minute pseudoplasmodia and subsequently produce diminutive fruiting structures.

Sorocarp formation in *Dictyostelium minutum* differs from *D. mucoroides* principally in the much smaller masses of myxamoebae that are involved. Sorocarps are invariably formed at the sites of aggregation and are regularly borne perpendicular to the substratum. While this may possibly result from an absence of light sensitivity, it is believed to be due to the small dimensions of the sorocarps (27). The fruiting organizations of *D. minutum* are from the outset diminutive; in fact, the limited number of myxamoebae that can effectively coöperate in fruit formation constitutes the very basis of the distinctive pattern which characterizes this species.

In *Polysphondylium violaceum* sorocarp formation follows the same course as in *Dictyostelium mucoroides* and *D. purpureum* except for one important difference. As the sorophore develops and the body of aggregated myxamoebae is raised above the substratum, masses of myxamoebae are periodically left behind while the ever decreasing body ascends the lengthening sorophore. Characteristically, these masses are deposited at comparatively regular intervals, and in general each succeeding mass is progressively reduced in size, although the first and second commonly present exceptions to this general rule.

As the stalk is extended and additional masses of myxamoebae are left behind, the one earliest deposited becomes vertically segmented and from each segment develops a side branch perpendicular to the main axis. Typically these are arranged in whorls, and the number and size of the branches depends upon the general dimensions of the whole fruiting mass and specifically upon the quantity of myxamoebae which remains at that level. Progressing upward, other masses segment and develop side branches. While I have not succeeded in obtaining as good photographs of this process as I would like, the essential steps are, I believe, shown in Figure 7, *A-C*. The number of whorls per plant and the number of branches per whorl depend upon several factors, most important of which is the quantity of myxa-

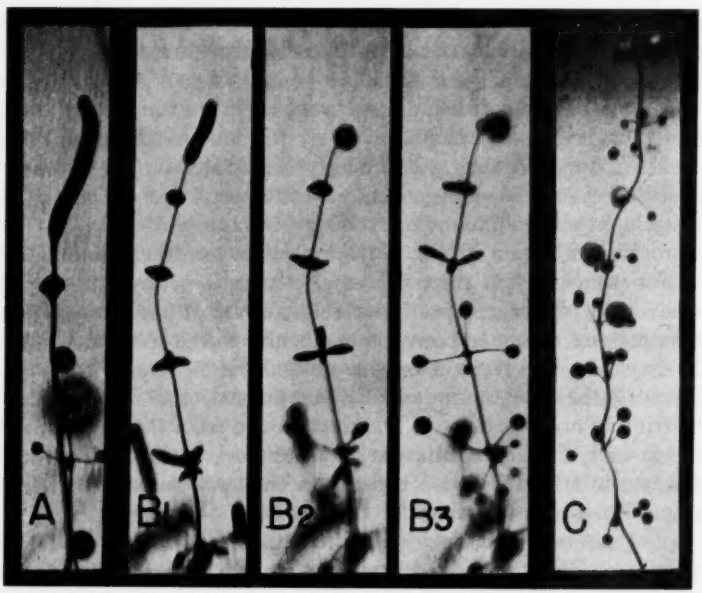

FIGURE 7

PROGRESSIVE STAGES IN SOROCARP FORMATION IN *Polysphondylium violaceum*

A, young sorocarp showing elongate terminal sorogen and the first deposited mass of myxamoebae. In the lower portion of the photograph are shown the terminal portions of two completed sorocarps. B_{1-3}, successive stages in the development of a single sorocarp; note particularly the development of the lateral branches in the two lower whorls; B_2, exposed 20 minutes after B_1 and B_3, 25 minutes after B_2. *C*, greater portion of a mature sorocarp showing general habit; upon touching the agar surface, sori immediately spread as shown at the fourth and fifth whorls. *A* and *B*, x 25; *C*, x 18.

moebae contributing to the formation of the sorocarp. The main axis, as in *D. mucoroides,* is characterized by a comparatively even taper and is terminated by a single sorus which is regularly somewhat larger than that borne upon any lateral branch. As Olive (16) and Harper (8) have pointed out, the formation of an individual branch duplicates the formation of the main stem, differing only in (*a*) its smaller dimensions, (*b*) the more pronounced taper of its stalk, and (*c*) its anchorage to the main axis rather than the substratum.

Sorocarp formation in *Polysphondylium pallidum* differs only in minor details. The sorocarps of this species are more delicate in pattern throughout. The number of whorls per plant and likewise the number of branches per whorl are, however, characteristically greater than in *P. violaceum* (Figure 1). In large sorocarps, characteristic of rich cultures, branches bearing whorls of branchlets are commonly seen. Whereas Olive (16) reported this only for a single strain, I have regularly encountered it in rich cultures of all isolations of this species.

For a complete discussion of sorocarp formation in *Polysphondylium,* the reader is referred to Professor Harper's detailed papers on *P. violaceum* (9, 10). In the present report I have endeavored only to relate the manner in which these relatively complex and symmetrical fruiting structures develop from integrated masses of undifferentiated and apparently similar amoeboid cells. As I have noted earlier, Bonner (2) has recently observed, by means of time-lapse pictures, periodic rhythmic contractions in the ascending mass of myxamoebae of *Dictyostelium discoideum.* While it can be proven or disproven only by similar time-lapse pictures, it is entirely possible that the regular branching habit of sorocarps of *Polysphondylium* may arise by a rhythmic advance of the main mass of myxamoebae, each time leaving behind a portion of the body which subsequently produces a whorl of side branches.

H. Sorocarp Size and Pattern

The ultimate size and pattern of any developing sorocarp is governed by both inherent and environmental factors. For every species, and to a less degree for each particular race or strain, there is a basic pattern which sorocarps attain, or approach, under optimal culture conditions. Likewise there is for each species a maximum size to which sorocarps can be constructed. In reality, however, these levels are commonly not attained.

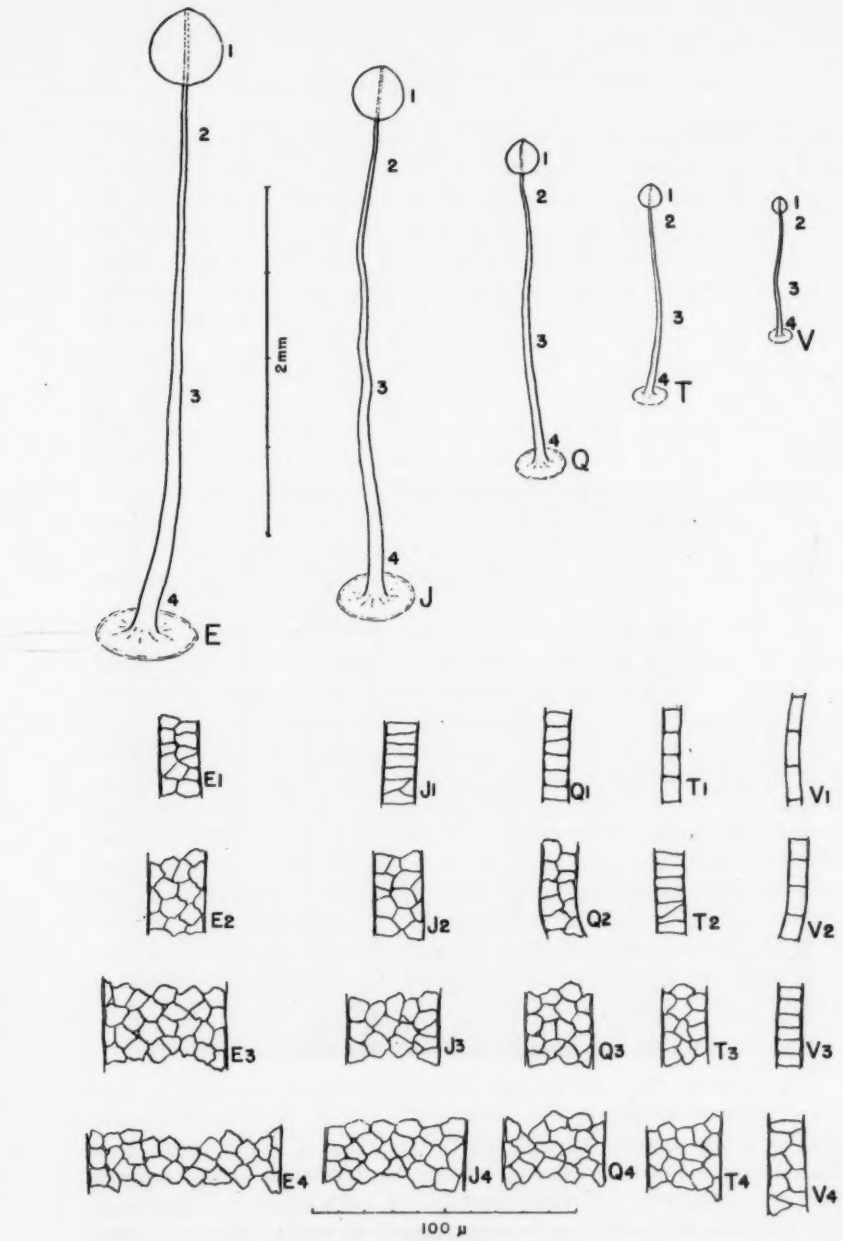

FIGURE 8

CAMERA LUCIDA SKETCHES OF MATURE SOROCARPS OF VARYING SIZE WITH DETAILS OF THEIR SOROPHORE CONSTRUCTION

E and *J*, large sorocarps; E_{1-4} and J_{1-4} sections of sorophores as indicated. *Q*, medium sorocarp; Q_{1-4}, indicated sections of the same. *T* and *V*, small sorocarps; T_{1-4} and V_{1-4}, sections of sorophores. Note particularly that the pattern of the sorocarp remains essentially constant irrespective of its size and that individual myxamoebae in contributing to stalk formation assume whatever shape is necessary to attain this pattern. The dimensions of these sorocarps are given in Table 1 under the same numbers.

TABLE 1

SIZE RELATIONS OF PARTS IN SOROCARPS OF DIFFERENT DIMENSIONS IN *Dictyostelium discoideum*

Sorocarp number	Sorus		Basal disk	Sorophore					Source	
	Diameter in μ	Volume in cu. μ	diameter in μ	Length in μ	Diameter in μ				Culture medium	Bacterial associate
					Base (4)*	Shaft (3)*	Top (2)*	Apex (1)*		
A	460	509×10^6	710	4500	112	70**	—	18	Lactose-Peptone***	*E. coli*
B	445	460×10^6	580	4250	110	70**	—	18	Lactose-Peptone	*E. coli*
C	420	387×10^6	560	4050	85	60	20	16	Endo's	*E. coli*
D	380	287×10^6	510	3900	82	55	18	14	Endo's	*E. coli*
E	380	287×10^6	460	3500	70	40	20	15	0.5% Peptone + PO_4****	*E. coli*
F	375	276×10^6	525	3750	80	45	20	15	0.5% Peptone + PO_4	*E. coli*
G	360	244×10^6	420	3900	85	45	20	15	0.5% Peptone + PO_4	*E. coli*
H	300	141×10^6	390	2975	52	35	16	12	Endo's	*E. coli*
I	300	141×10^6	410	3300	72	38	18	12	0.1% Peptone + PO_4*****	*B. megatherium*
J	280	114×10^6	410	3000	50	35	16	10	0.5% Peptone + PO_4	*E. coli*
K	240	72.3×10^6	360	2750	50	35	18	10	0.5% Peptone + PO_4	*E. coli*
L	225	58.3×10^6	330	2400	48	30	18	8	0.5% Peptone + PO_4	*E. coli*
M	210	47.4×10^6	240	2500	58	35	16	11	0.1% Peptone + PO_4	*B. megatherium*
N	210	47.4×10^6	300	2700	42	28	12	8	Endo's	*E. coli*
O	180	30.5×10^6	270	2000	40	26	10	8	0.5% Peptone + PO_4	*E. coli*
P	175	28.0×10^6	240	2460	60	32	14	12	0.1% Peptone + PO_4	*B. megatherium*
Q	155	19.5×10^6	235	1750	40	26	12	7	0.5% Peptone + PO_4	*E. coli*
R	150	17.6×10^6	210	1800	38	25	12	7	Endo's	*E. coli*
S	150	17.6×10^6	215	1640	45	28	12	10	0.1% Peptone + PO_4	*B. megatherium*
T	120	9.0×10^6	180	1200	28	16	10	6.5	0.5% Peptone + PO_4	*E. coli*
U	120	9.0×10^6	160	1500	32	20	10	8	0.1% Peptone + PO_4	*B. megatherium*
V	65	1.4×10^6	105	750	12	7.5	5.5	4.5	0.5% Peptone + PO_4	*E. coli*

*Positions measured are designated in Figure 8 by these numerals.
**Diameter approximate—Sorophores measured 60 μ at mid-point.
***Medium containing 1.2% each of lactose and peptone.
****Medium containing 0.5% peptone + M/50 KH_2PO_4 and M/50 $Na_2HPO_4.12\ H_2O$
*****Medium containing 0.1% peptone + M/100 KH_2PO_4 and M/100 $Na_2HPO_4.12\ H_2O$
Sorocarps *E, J, Q, T,* and *V* are illustrated in Figure 8.

Let us consider *D. discoideum*. In this species, as in all the larger forms, sorocarps vary enormously in size even within the same culture. In very rich cultures the number of large sorocarps is substantially increased; but irrespective of their number, the dimensions of individual fruiting structures do not exceed those of sorocarps *A* and *B* in Table 1; in fact, they rarely attain them. On the other hand, there is no minimum size, and fruiting structures of even smaller dimensions than listed for sorocarp *V* in Table 1 and Figure 8 are not infrequently seen. Between these two extremes, sorocarps of every intermediate size may be found, as is indicated in Table 1. Under favorable circumstances a large percentage of the sorocarps generally fall within the range between sorocarps *F* and *Q* of Table 1. The formation of small sorocarps may result either from (*a*) a scarcity of myxamoebae resulting in the formation of small pseudoplasmodia originally, or (*b*) the break-up of large pseudoplasmodia (radiate or migrating) into smaller organizations prior to the beginning of sorocarp formation. Factors influencing pseudoplasmodium size (e.g., temperature, humidity, and light) are thus seen to govern directly the size of resulting fruiting structures.

Regardless of its dimensions, however, the sorocarp of *Dictyostelium discoideum*, if formed under favorable circumstances, is of essentially the same pattern. As noted earlier, and as illustrated in Figure 8, each consists of an upright, tapering sorophore rising from an expanded basal disk and bearing at its apex a spherical to slightly elliptical spore mass. The essential thing is not the size of the fruiting structure but its pattern, just as the essential thing about a pseudoplasmodium is not the number of myxamoebae comprising it but the manner in which these individuals are organized. The capacity to form coöperatively such a fruiting structure obviously represents an inherent species character, and while this capacity is lodged in the contributing myxamoebae individually, it depends for expression upon the integration and coördinated differentiation of these multiple units. While sorocarp patterns are definitely inherited just as are the distinguishing characteristics of higher plants, we must not lose sight of the fact that we are dealing with what one may possibly term "race inheritance" rather than inheritance in individuals. What is inherited is not a specific type of structure, but the ability of like but discreet and independent units to coöperate in the formation of such a structure. The best evidence of an inherited capacity of myxamoebae to fruit only with other individuals of their own kind is seen in cultures

where two species are intermixed during their vegetative phase. Two species of *Dictyostelium* may initially enter the same fruiting organization, but they subsequently draw apart and form separate sorocarps (26).

While it is impossible to say just what is responsible for the consistent pattern of the sorocarps of a particular species, in *Dictyostelium discoideum* it is believed that the sorophore sheath represents the principal means by which this is accomplished. The appearance of the hyaline tube represents the first step in sorocarp formation, and while its exact origin is not known, we do know that it is the product of many cells and represents a function of the entire body (p. 59). In sorophore development this sheath is formed in advance of cellular differentiation, and, in fact, prescribes the limits within which such differentiation must take place. As the body of myxamoebae ascends the elongating sorophore, its mass becomes progressively reduced as part of the myxamoebae contribute to stalk formation and others become reduced in size incident to spore maturation. This continuously decreasing volume is reflected in the progressively smaller diameter of the lengthening sheath. The final result is that the proportions, and therefore the pattern, of the sorocarp are maintained, while individual cells contributing to stalk formation assume whatever shape is necessary to accomplish this result. This condition is very pronounced and is clearly shown in the smaller sorocarps illustrated in Figure 8. If the diameter of any portion of the sorophore is greater than 12μ, it consists, in cross section, of a number of essentially isodiametric cells (Figure 8, E_1), the number of such cells being proportional to its cross sectional area; if it is approximately 10μ in diameter it consists of a single tier of horizontally flattened cells (Figure 8, J_1 and T_2); if 7-8μ in diameter the single row of cells are approximately isodiametric (Figure 8, Q_1); whereas if the stalk is less than 7μ in diameter the cells are vertically elongate and the smaller the stalk diameter the more elongate these become (Figure 8, V_1). For *D. mucoroides* and *P. violaceum* sorocarp size and cell shape relationships have been reported in considerable detail by Harper (8, 9).

Although the sorocarp is formed by the differentiation of individual cells, its form is obviously determined by the collective but unitary action of the whole mass of myxamoebae. The relationship is by no means an exact one, but, as evident from Table 1, the diameter of the sorus is generally about 1/10 - 1/12 the length of the stalk, whereas the diameter of the stalk base is roughly 1/4 - 1/5 the diameter of

the sorus. It is primarily this general size relationship of parts which gives to the sorocarp of this species its distinctive character and pattern.

Sorocarps of typical and distinctive pattern, but of variable size, likewise develop regularly in other species under favorable culture conditions. In the ubiquitous species *D. mucoroides,* even particular isolations maintain characteristic structural differences during years of continuous laboratory cultivation. The typical sorocarp of *D. mucoroides* may be said to consist of a comparatively long and slowly tapering sorophore bearing at its summit a milk-white sorus of proportional size. Branches, when present, are small and irregularly spaced. Yet strains have been isolated which, under comparable conditions, consistently produce sorocarps characterized by (*a*) relatively longer and thinner sorophores, (*b*) relatively shorter and heavier sorophores, or (*c*) more commonly branched sorophores. The most distinctive strains of *D. mucoroides,* however, are those occasionally isolated in which certain pseudoplasmodia, instead of forming normal sorocarps, round up and become surrounded by comparatively thick, hyaline walls forming cyst-like bodies. Since these have not been reported by subsequent authors it is of particular interest that in his paper describing this species Brefeld (3) illustrates such bodies, terming them dwarf sporangia. Strain variations are not so well marked in *D. purpureum* as in the above species, but isolates persistently producing sorocarps of somewhat different proportions have been encountered. To a less degree this is likewise true of *Polysphondylium violaceum* and *P. pallidum.*

Whereas sorocarps are regularly typical in pattern when formed under favorable environmental conditions, they are characteristically quite atypical when formed under less favorable circumstances. For example, the effect of *pH* upon the fructification of *D. discoideum* is most striking. Within the range of *pH* 5.0 to 7.0 sorocarps of normal pattern are regularly produced; at *pH* 7.5 sorocarps are characterized by short, heavy sorophores, poorly formed basal disks, and comparatively large sori; at *pH* 8.5 little or no growth occurs and sorocarps are not produced (22). The production of similar abnormal fruiting structures can likewise be induced by incubating cultures somewhat above the optimum temperature range (20° - 24° C.) for the species, or by introducing dry air into the culture chambers during the period of sorocarp formation. Although detailed studies of the influence of these factors upon sorocarp formation in other species have not been

made, it is my experience that sorocarp patterns in these forms are affected to a comparable degree if not in a strictly comparable manner.

Light commonly determines the location of sorocarps of *Dictyostelium discoideum* but it does not alter their basic pattern. In such long stalked forms as *D. mucoroides, D. purpureum,* and *P. violaceum,* however, the effect of light upon sorocarp pattern is frequently very striking. Not only are sorocarps regularly smaller and more numerous in well illuminated cultures than in similar ones incubated in darkness as reported by Potts (19) and Harper (10), but also the ratio of stalk size to spore mass is often materially increased. This can be readily demonstrated by exposing like cultures to full and direct illumination and to weak illumination entering the culture from one side. Under the former conditions sorophores are commonly 1-1.5 cm. in length and bear sori 300-350μ in diameter. In the latter cultures, sori of equal size are commonly borne on sorophores of approximately the same diameter but 4-5 cm. in length. Essentially the same response may be observed in *Polysphondylium violaceum,* but because of the repeated production of lateral branches it is more difficult to evaluate. Sorocarp pattern in *P. pallidum,* on the other hand, appears to be only slightly influenced by light.

It is thus apparent that while there exists for every species a typical sorocarp pattern and a maximum sorocarp size, commonly these are not attained. Frequently this results from environmental factors and the importance of maintaining optimal cultures when studying the development of these slime molds cannot be overemphasized.

I. General Considerations

What I have reported up to this time constitutes, in the main, a part of the published record concerning the behavior and development of these slime molds. Now, in conclusion, I should like to pose some questions which, when properly answered, will contribute greatly to our knowledge of these interesting organisms. These questions fall into four groups, and in each case I shall list first the related questions and subsequently review the limited information we have concerning them.

1. *Why do the myxamoebae come together? What is the stimulus responsible for their aggregation and how is this transmitted? What determines the position of aggregation centers?*

The aggregation of myxamoebae into pseudoplasmodia constitutes, I believe, the most fascinating phase of development in these slime

molds. It has engaged the attention of all students in this field and yet the basic cause, or causes, responsible for it remains imperfectly understood. Olive (16) attributed the aggregation of myxamoebae to chemotactic stimuli originating in the mass of centrally placed individuals and diffusing outward through the culture medium. Potts (19) likewise concluded that aggregation was due to stimuli of chemotactic nature. While neither investigator succeeded in identifying the agent, or agents, responsible for aggregation, the progressive orientation of myxamoebae outward from definite centers certainly reveals the basis for their reasoning.

We know that the aggregation of myxamoebae is in some way associated with a food shortage, as has been reported by numerous investigators (19, 14, 28, 1, 25). In *Dictyostelium discoideum* pseudoplasmodium formation normally does not occur until the available bacteria have been consumed. By adding fresh bacteria to a vegetative culture, aggregation can be substantially delayed, and by transferring myxamoebae to fresh bacterial colonies at intervals up to 36 hours, cultures can be maintained indefinitely without showing any fruiting phenomenon (25).

Working with *Dictyostelium mucoroides,* Arndt (1) concluded that food shortage was the indirect rather than the direct cause of pseudoplasmodium formation. The latter he attributed to changes which take place within the myxamoebae when inadequately nourished. He believed the stimulus responsible for aggregation arose from within the myxamoebae themselves and varied in intensity in different groups of individuals and in time and place. In support of this explanation he showed that in the initial phase of pseudoplasmodium formation, termed the *pre-plasmodium,* temporary centers of orientation, designated *cumuli,* arose which were neither fixed in position nor constant in the number of myxamoebae subservient to them. The *cumuli* were generally short-lived, disappearing and re-appearing in other locations until some center of aggregation gained the ascendancy and henceforth controlled the convergence of myxamoebae in the surrounding area. From his film covering this stage, Arndt found that the stimulus responsible for aggregation varied in intensity, thus producing waves which effect rhythmic responses and contractions in the aggregating mass. More recently, and now reported for the first time, Bonner (2) demonstrated a comparable wave motion in *D. discoideum* in the process of sorocarp formation. It would appear that this represents a fundamental characteristic of these slime molds, and a further

elaboration of this phenomenon may go far toward explaining the method of stimulus transfer and possibly the specific nature of the stimulus itself.

In conversation, Professor Harper has likewise reported observing a preliminary aggregation stage in *Dictyostelium mucoroides,* but insofar as I am aware, his observation remains unpublished. As I have already noted, in my own experience the establishment of definite aggregation centers is not always the first evidence of pseudoplasmodium formation, although this is generally true. In the present case, as in others, I suspect it is the exception which proves the rule. Certainly the establishment of movable and non-persisting centers of aggregation indicates that the responsible stimulus has its origin within the myxamoebae. That this is true is likewise indicated by the fact that the stimulus responsible for aggregation varies qualitatively in different species, and particularly in different genera (26). The myxamoebae of *D. discoideum* and *Polysphondylium violaceum,* for example, although planted together and thoroughly intermixed throughout their vegetative stage, aggregate to separate and distinct centers. This would hardly be expected if aggregation were due to a negative hygrotropic response as has been maintained by Schuckmann (28) and Harper (8), or to some other factor arising from the physical environment.

Although there is no indication that these factors are responsible for aggregation, it may be noted finally that increased temperature (from 24° to 28° C.), decreased humidity (exposure of cultures to dry air), and incubation in light hastens the initiation of aggregation from 2 to 4 hours (25).

2. *How does the migrating pseudoplasmodium move? By what means is the movement of its constituent members translated into a movement of the whole body?*

The locomotion of the migrating pseudoplasmodium presents an interesting problem and one concerning which we have only limited information. I have previously noted that it consists of a large but variable number of undifferentiated myxamoebae surrounded by a non-cellular sheath, or envelope. Since the pseudoplasmodium represents a community of individual myxamoebae it is obvious that the movement of the whole mass must result from the coördinated action and movement of the multiple units which comprise it. There is, however, the problem of how their individual movements can effect a unitary movement of the whole body. As a means of approaching

this problem I shall suggest certain possibilities, and then consider what evidence we have in support or refutation of each. *First,* the whole mass would move forward if the myxamoebae within it moved in a cycle comparable to the movement of the cytoplasm within the body of an amoeba, i.e., by the continual movement of individuals from the posterior-most portion of the body to its anterior end. That this does not occur can be demonstrated in a number of ways, the most striking of which is to graft a red apical fraction upon a colorless body (25). If there was a movement of individual cells, as postulated above, either the whole body would become pink or the posterior end only would be red after the body had migrated its own length. As a matter of fact neither occurs; the anterior tip remains red even to the completion of sorocarp formation, proving beyond question that the same myxamoebae continually occupy an anterior position in the pseudoplasmodium. *Second,* the whole mass would move forward as a unit if each myxamoeba moved forward at an approximately uniform rate. Likewise this would permit individual myxamoebae to occupy the same relative positions in the pseudoplasmodium during extended periods of migration. Just how this can be accomplished, however, is difficult to comprehend. If all of the myxamoebae move at a uniform speed the problem of traction for the uppermost individuals becomes decidedly interesting, for it is estimated that a line from the upper to the lower surface of a pseudoplasmodium 200μ in diameter would transect between 25-30 myxamoebae, one above the other. *Third,* unitary movement of the pseudoplasmodium likewise would be effected by the coördinated movement of the bottom layer of myxamoebae only, these in effect carrying the multiple layers above them. This, however, would place a heavy and possibly unmovable load upon the layer of propelling individuals. *Fourth,* the occasional spiral pattern of the migrating body suggests a type of intra-pseudoplasmodial movement of individuals which may bring about a movement of the whole body. This would entail the differential but coördinated movement of relatively localized groups of myxamoebae in a definite pattern or cycle, so that the separate movements of these groups would be cumulative. While this concept remains vague, even in my own mind, it seems to suggest an explanation consistent with the observed facts of pseudoplasmodial movement.

That the pseudoplasmodium does not move as a semi-rigid body by some force aside from that of the multitude of myxamoebae which comprise it is obvious. If the body of a pseudoplasmodium is pierced

by a series of thin glass needles (25μ $\pm$ in diameter), and these are securely anchored in the underlying agar, the pseudoplasmodium flows by them as if it were a fluid mass and at an apparently unslackened rate.

3. *Wherein are the governing centers of the migrating pseudoplasmodium lodged? How do these centers arise and how is their influence transmitted through the body?*

The governing centers of the migrating pseudoplasmodium are located near its apical end, and are presumably lodged within a group of strategically placed myxamoebae. As previously reported (25), it is not known whether these individuals arrive at such a position because of special capacities or whether they assume special capacities because they occupy a strategic position. There is evidence that the centrally placed myxamoebae in the developing pseudoplasmodium subsequently constitute the apical portion of the migrating body and that they become increasingly specialized in function. When the central portion of a developing aggregate is removed, other centers capable of directing migration subsequently arise; but when the apical portion of a migrating body is removed further migration of that body is normally precluded.

I have previously reported that the directive and receptive centers were lodged somewhere within the anterior 1/8 or 1/10 of the migrating pseudoplasmodium, for by removing this portion the migration of the body, except in isolated cases, was terminated (25). Recently I have attempted to define more exactly the position of the controlling centers. The results are inconclusive. In one experiment, anterior tips amounting in each case to approximately 1/20 of the total mass of the pseudoplasmodium were removed. In 24 out of 30 cases the decapitated pseudoplasmodia promptly initiated sorocarp formation. The remaining six, however, subsequently developed new centers and resumed migration. In another experiment, I removed from 20 large migrating bodies minute apical fractions, in each case amounting to approximately 1/100 of the total mass by volume. A similar number of like but unmolested pseudoplasmodia were marked as controls. In only 3 of the 20 cases was migration terminated and sorocarp formation begun. In the remaining cases, however, there was generally present in the slime streak evidence pointing to a temporary cessation of migration (25, p. 257). In none of the controls was migration hindered even temporarily. Thus it appears that we may be approaching a critical level in the pseudoplasmodium. The temporary cessation

of migration would seem to indicate that the governing centers were removed from each pseudoplasmodium, and that in three instances the bodies were unable to re-form such centers whereas in the remaining cases they succeeded in re-establishing them. While I have worked with the greatest care possible under existing circumstances, I wish to point out that in order to perform conclusive experiments of this nature one should work in a room at constant temperature containing a saturated or near-saturated atmosphere. Under these conditions the opening and closing of culture dishes should be without effect.

4. *What determines the ratio of spore and stalk forming myxamoebae in the pseudoplasmodium? What can account for the formation of normal sorocarps by posterior fractions of a migrating pseudoplasmodium?*

The myxamoebae comprising the apical portion of the migrating pseudoplasmodium (approximately 1/3-1/4) normally contribute to the building of the sorophore, while the remainder forms the sorus and basal disk. This can be easily demonstrated either by grafting a red apical fraction upon a decapitated colorless body, or by removing an apical fraction and forcing it alone to fruit immediately. In the former case a sorocarp of normal pattern but characterized by a red stalk is produced, whereas in the latter case a sorocarp of abnormal pattern is formed in which the stalk is exceptionally heavy, the basal disk absent or poorly formed, and the sorus lacking or abortive. These and comparable experiments clearly demonstrate that the myxamoebae of the anterior portion are not only destined to form stalk cells but are, in fact, already somewhat specialized in that direction. What they fail utterly to reveal is the source of the stalks formed from posterior fractions which normally would have contributed only to the formation of spores. Let us go back to the apical fraction. If this is permitted to migrate for several hours before forming a fruiting structure, a sorocarp of essentially normal proportions is produced, indicating that cells at one time intended only for stalk formation retain a capacity of functional readjustment. The specialization of myxamoebae thus appears to be uni-directional. Individuals once destined to become stalk cells can gradually regain their capacity to produce spores, while myxamoebae normally destined to produce spores never lose their capacity to produce stalk cells.

J. Conclusion

I have endeavored to summarize, insofar as is known, the developmental history of the Dictyosteliaceae and to show the manner in which sorocarps of consistent and specific patterns arise in different members of this group. Concerning certain phases of this process many questions remain unanswered. In addition to these, there are questions of broader significance which concern the relation between certain of these phenomena and vital processes exhibited by other organisms. For the study of such relationships and processes, it is my belief that these simple slime molds afford exceptionally useful and productive tools.

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GROWTH PATTERNS IN PLANTS

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It was with some hesitation that I accepted the invitation to present a paper at this conference on growth. I have little to contribute in the field of growth patterns except problems and some of these I shall be glad to outline with the hope of getting suggestions for their solution. At the present time we seem to be going through a profitable stage of coöperative attack on broad problems of science by means of group conferences which are more or less informal in nature and give opportunity for leisurely discussion of matters of common interest. It has occurred to me that almost any of the papers presented this summer at the conferences at Gibson Island on chemical problems, at the Plant Hormone Conference in New London, or at the symposium which I have recently attended in Cold Spring Harbor on the structure of genes and chromosomes would be found to have a bearing on the problems we are discussing here. I feel sure I could outline a full curriculum embracing all of biology which would be based on behavior of chromosomes and could properly be labelled Genetics and there is little doubt that the taxonomist, physiologist, or chemist would be equally fertile in building up a classification of science which would center about his own special field of work. All these different points of view are of value in a study of any aspect of life. It is with this thought that I shall say something about growth patterns from the standpoint of a student of chromosome behavior.

CHROMOSOMAL TYPES

Chromosomes can be considered from two aspects: first, as the treasury of the biological wealth of the past—in other words, as the bearers of hereditary characters; and secondly, as continuing inducers of changes in environment through interaction with existing environments which may be internal as well as external. We can recognize the final external pattern in the various organs of a plant, and can see the chromosomes in the germ cells and follow their behavior in division of cells, but we know all too little of the processes that are taking place between the germ cells and the finished organs. It is clear that individual chromosomes do not bring about characters

without aid. Their influence is through interaction with the external environment on the one hand and through interaction with the influence of other chromosomes on the other hand. The pattern of the plant is determined by continuing interaction of many factors. When one is a limiting factor we often speak of it as the "cause" of the particular pattern but this is justified only if the influence of all the other factors remains unchanged. The interaction of chromosomes on growth pattern may be seen from photographs of capsules of the 12 primary $2n+1$ types in Datura (5). Each of its 12 chromosomes when present as an extra has a specific influence upon the form of capsule which makes it different in appearance from that of a normal $2n$ plant. When two different extra chromosomes are present the capsule shape is the resultant of the forces exerted by the two extra chromosomes. Sometimes one chromosome may appear dominant in respect to certain of the characters of a double $2n+1+1$ type and the other chromosome may appear dominant in respect to certain other characters. Other characters may appear to be intermediate between those induced by each chromosome when present as an extra in $2n+1$ types. A similar interaction of influence upon characters of capsules and other organs of Datura may be seen in related secondary types in which the extra chromosome consists of one half doubled. Thus the $2n+1{\cdot}2$ primary type has the related secondaries $2n+1{\cdot}1$ and $2n+2{\cdot}2$. It can be seen from photographs that the $1{\cdot}1$ chromosome contains factors which tend to make the capsule small and narrow with slender spines, while the $2{\cdot}2$ chromosome contains factors which cause the capsule to be large sugar-loaf shaped and to have short stout spines. Since the primary $2n+1{\cdot}2$ type has both an extra $\cdot 1$ half and an extra $\cdot 2$ half, the capsule shape is intermediate between that of its two secondaries, each of which it resembles in some respects. The influence of an extra chromosome depends upon the basal chromosomal constitution to which it is added. Thus it can be shown that the extra $21{\cdot}22$ chromosome in a $2n+21{\cdot}22$ type which has a numerical unbalance of 1 vs 24 (the $2n$ number of Datura) has a strong influence in depressing the capsule and producing stout basal spines. The extra chromosome in a $4n+21{\cdot}22$ has only a relatively feeble effect upon the capsule shape since it has a lessened unbalance of 1 vs 48. The addition of two extra chromosomes to a tetraploid to form a $4n+2(21{\cdot}22)$ produces an unbalance of 2 vs 48 which is numerically equivalent to the unbalance of 1 vs 24 in a $2n+21{\cdot}22$ type and the capsules of these two types are equally distinct from the types to

which the extra chromosomes were added. They are not alike in appearance, however, since in one case the basal chromosome number is 24 ($2n$) and in the other 48 ($4n$) and the $4n$ capsule is more nearly spherical than the egg-shaped capsule of a diploid.

All told there has been accumulated a considerable collection of unbalanced chromosomal types in Datura (9) which emphasize the interaction of whole chromosomes and parts of chromosomes. Sinnott in a coöperative study (16) of the internal anatomy of such forms finds that distinctive tissue patterns are associated with particular extra chromosomes. The extra chromosomal material may alter the number or size of cells. Anatomical pattern as shown by the relative local development of the various tissue systems or by the shape of such structures as the vascular bundles is also specifically determined by the various chromosomes, and such traits seem to be controlled independently of those of general size and number. The fact that certain of the anatomical differences studied are evident in the earliest developmental stages and others do not appear until the latest ones, suggests that the chromosomal control of the rates of various developmental processes may provide the genetic mechanism whereby certain anatomical differences are determined.

We have spoken of the changes in pattern induced by an unbalanced chromosome constitution. Changes are also induced when the number of chromosomes is altered without change in the numerical balance. Thus a distinct increase in size of flowers of Datura (4) is seen as one goes from the $1n$ through $2n$ and $3n$ to the $4n$ condition. This is the general rule but in a few plants (the garden Portulaca, for example) $4n$ flowers cannot be readily distinguished by size alone from $2n$ (6). In Datura there are qualitative differences among the balanced polyploids. Thus the leaf of the $1n$ is relatively narrow and the width increases with increase of chromosome number to the $4n$. Similarly the capsule becomes shorter and stouter as the balanced chromosome number increases. Why a $4n$ leaf should be broader than that of a $2n$ or why a $4n$ should be more nearly spherical than a $2n$ capsule is not entirely clear. It may be a matter of differences in thresholds. Doubling the number of certain of the chromosomes may have greater effect on pattern than doubling others.

Fruit Shape in Cucurbits

Interaction of chromosomes is seen in the differences between $2n$ and $4n$ fruits brought out in a coöperative study with Sinnott of the

effect of polyploidy upon pattern changes in different species of cucurbits (7, 15). The 4*n* fruits of the Spoon gourd (Cucurbita) are larger than the 2*n* fruits but keep the same general shape. The width is increased only slightly if at all more than the other dimensions. This is not at all true of the Club gourd. In this species the 2*n* fruits are narrow and long and may reach a length of 5 or 6 feet. The 4*n* fruits are actually considerably shortened and the width is greatly increased. This change in shape is in accord with the usual effect of tetraploidy in other forms in which the fruits of the tetraploid may be generally described as stouter than diploid. Doubling the chromosomes of one of the Pear-shaped gourds (Cucurbita) causes the fruits to be disc-shaped. This effect of tetraploidy is of interest since Sinnott has found that the difference between pear-shaped and disc-shaped fruits is in diploids determined by a single pair of genes. The Bottle gourd (Lagenaria) has a distinct neck with a constriction at the lower end where it connects with the nearly spherical basal portion. In the fruit of the 4*n* Bottle gourd the neck is entirely eliminated and the basal portion is much shortened. From these examples it will be seen that the different pattern changes brought about in the 4*n* fruits of cucurbits depend upon the different chromosome constitution of the 2*n* species before the chromosome number is doubled. Sinnott has found that differences exist between species also in the effect of tetraploidy upon relative size of cells (15). In the small round China gourd (Cucurbita) the cells of the epidermis, of the fruit wall and of the pith he finds larger in the 4*n* fruit when it is only 3 mm. long. In this gourd the greater size of 4*n* over 2*n* cells in these tissues continues throughout growth till maturity. The condition is quite different in the Sugar Trough gourd (Lagenaria). In this gourd the 4*n* cells of the three tissues under discussion are larger than those of 2*n* cells when the fruits are young but the increase in size is more rapid in the 2*n* cells so that as the fruits mature the 2n cells become actually larger than the 4*n* cells of the corresponding tissues.

Periclinal Chimeras

We have been discussing the effects of tetraploidy upon growth patterns. The assumption has been general that when tetraploidy is induced, an exact doubling of the chromosome number takes place in all tissues of the plant. This is very far from the case. We have long known that when tetraploids arise in Datura they are usually

rough-leaved mixochimeras, a mixture of chiefly $2n$ and $4n$ tissue. Ultimately branches may grow out that may be pure $2n$ or $4n$ and the offspring from the latter may give rise to $4n$ races. Colchicine (8) not only induces doubling of the chromosome number but causes elimination of individual chromosomes to produce a series of $2n$—1 and $4n$—1 types of characteristic appearance in which the effect of chromosome deficiences on growth patterns can be studied (1). When seeds are treated with X-rays or other stimuli to gene mutations only one of the cells in the growing point will undergo a particular kind of mutation which will affect all its descendant cells in a sector of the adult plant. The commonest kinds of mutation are those which cause abortion of the pollen grains which carry the mutant gene (11). By examining the pollen of flowers on different parts of the plant it is possible to determine how large a sector has developed from a particular kind of pollen abortion mutation. The evidence is clear that only a few initial cells in the seed take part in the development of the shoot. The most frequent condition when such mutations are present is to find one half of the plant affected. In some cases the whole plant shows the same pollen abortion mutant type—a fact which with other evidence would indicate that such a shoot (at least the part which produces pollen grains) had come from the development of a single initial cell. Occasionally the mutated sectors are small. In one case we had evidence that at least five initial cells had taken part in formation of the shoot since there were five different kinds of sectors present as judged by the condition of the pollen or the character of the offspring from different capsules.

Colchicine is effective in doubling chromosome number only when the nuclei are in division. It is evident therefore that only one or a relatively few of the initial cells in a seed may have its chromosome number doubled from a single treatment. It is also evident that if different germ layers are present in the seed it may happen that the doubling of chromosome number will be confined to only part of the germ layers. This has been found to be the case in Datura. Among several thousand plants which came from colchicine-treated seeds there were frequent instances in which the records of the plants from their appearance in the field did not agree with the determinations from size of pollen grains or of guard cells of the epidermis. A careful study of such cases by Miss Satin has shown that following treatment with colchicine there is frequent production of periclinal chimeras in which one layer only may have the doubled chromosome

number. The doubled number has been determined by actual count of the chromosomes but in cases in which the number has been doubled twice to form 8*n* cells, the enlargement of the affected cells is so great that the approximate number may be determined by size of the cells alone. A series of four longitudinal sections of the vegetative growing point furnishes evidence regarding the germ layers (7). The first is of a normal 2*n* control. There are about half a dozen more or less distinct layers evident with the cells and their nuclei of about the same size. The second section is of a periclinal chimera in which the first or outermost germ layer which forms the epidermis is 8*n*. The relatively enormous size of its 8*n* cells and nuclei clearly distinguish this single layer of cells from the others in the section. The third section is of a periclinal chimera in which the second germ layer is 8*n*. This is composed of a single layer of cells and gives rise by later divisions to the egg cells and pollen grains. The fourth section is a periclinal chimera in which the third germ layer is 8*n*. This by divisions gives rise to the central cylinder. It may appear to consist of several layers but in over two hundred periclinal chimeras studied, the innermost cells were obviously derived from the third germ layer since in all cases they had the same chromosomal constitution. The evidence seems clearly to indicate, therefore, that there are three and only three germ layers in Datura through the interaction of which the growth patterns in the plant are established.

Polyploidy in periclinal chimeras may be used like tracer elements to label the different germ layers and to determine the contribution which each makes in the development of a given organ. Thus it may be of help in a study of the homologies of the floral organs. Periclinal chimeras show that the petal and sepal are homologous with the leaf in that their primordia are alike in having the bulk of the cells made up from the second germ layer. The primordium of the stamen, however, is unlike that of the leaf in that the second germ layer consists of only a single layer of cells and the third germ layer forms the bulk of the primordium. In its development therefore the stamen appears to be homologous with the shoot rather than with the leaf, although the latter is the classical interpretation. Periclinal chimeras show how the first germ-layer or epidermal tissue may become internal. The petals are joined together in Datura to form a tubular corolla. Similarly the sepals in the adult flower are united to form a calyx cup. In the young flower, however, the petals and sepals are both separate. What happens in their union is very clear from a 2*n*, 8*n*, 2*n*

periclinal chimera. (The three germ layers are given in order from the outside in.) A cap of $2n$ epidermal tissue forms at the end of each petal. Their tips fuse forming a mass of internal $2n$ tissue at the region of union with the body of the petal which is made up of $8n$ cells of the second germ layer. This interpretation can be readily checked in an $8n$, $2n$, $2n$ periclinal chimera in which the internal tissue at the region of petal fusion is composed of $8n$ cells which come from the epidermal layer. A similar history occurs in the fusion of tips of sepals to form the calyx cup.

The three germ layers make different contributions to the growth pattern. This can be readily seen by comparing $2n$ and $4n$ capsules with those of the periclinal chimeras $4n$, $2n$, $2n$; $2n$, $4n$, $2n$; and $2n$, $2n$, $4n$. The third germ layer has most effect upon the shape of the capsule probably because the third layer forms the largest part of its internal tissue. The second germ layer has next greatest effect and the first germ layer has the least effect. A $2n$, $2n$, $8n$ capsule is much more depressed than a capsule which is $4n$ throughout.

It was somewhat of a problem to us why a $4n$, $2n$, $2n$ chimera was able to set capsules and seed from selfing while a $2n$, $4n$, $2n$ plant could not. The reason was clear when Miss Satin discovered from study of periclinal chimeras that the central strand of tissue within the style through which pollen tubes grow is derived like the epidermis from the first germ layer. The pollen tubes from a $4n$ plant Buchholz (10) has found usually swell up and burst when growing in a $2n$ style while pollen from a $2n$ plant grows readily in a $4n$ style. Since pollen comes from the second germ layer, selfing a $4n$, $2n$, $2n$ chimera is equivalent to the successful cross $4n \times 2n$ whereas selfing a $2n$, $4n$, $2n$ chimera is equivalent to the incompatible cross $2n \times 4n$, so far as pollen-tube growth is concerned.

The effect of these two periclinal chimeras on growth patterns of the pollen tubes indicates that it is the second layer alone which inhibits pollen-tube growth in the cross $2n \times 4n$. Illustrations of periclinal chimeras are given in a series of papers already published (7, 14) or in press (12).

Gene Mutations

Spontaneous mutations in genes are relatively rare in Datura but may be readily induced by radiation, heat, and aging seed. We now have nearly 500 types which we classify as probably due to altered genes. Some, such as the genes for white flowers, are of little or no

importance to the plant and for this very reason are good labels with which to tag the chromosomes in genetic tests. Others such as the genes for non-flowering, male and female sterility are disadvantageous to the plant and make poor genetic labels. Some, such as the gene which changes the dicarpel to a tricarpel ovary, and the gene which changes the black to red seeds, alter characters which have been used to distinguish species and even genera in the taxonomic group to which the Jimson Weed belongs. One type called "wilt" since it wilts in sunlight despite an abundance of moisture in the soil has been found by Sinnott to have a poorly developed fibro-vascular system which appears inadequate to meet the demands of rapid transpiration in sunlight. Among the commonest visible mutant types are those with some type of chlorophyll deficiency. If the type is unable to survive the cotyledon stage and dies in the seed pan we call it for convenience an albino. With care albino seedlings can be grafted and will produce an abundance of pale leaves and even set seed if the stock is allowed to form leaves which carry on photosynthesis for the parasitic scion. In one case a weak pale type which is barely able to survive when on its own roots grows vigorously and has normal green leaves when grafted on a normal stock. The inhibition to growth and formation of normal chlorophyll in this case may be localized in the root system of the pale mutant.

To us one of the most interesting mutant types is one which resembled the effects of a particular virus disease. Many years ago we found a type which was first thought due to a gene mutation but which later turned out to be due to a virus disease which may be propagated from one Datura plant to another by grafting but not by injecting juice of the diseased plant (2, 3). We called it the "*Quercina*" disease on account of its eroded leaves which resemble somewhat those of certain oaks. As the infection spreads through the plant the capsules become smooth and the flowers have separate petals. This last year from X-ray treated seed a recessive mutant type appeared which closely resembled the effects of the *quercina* disease in the erosion of its leaves, the lack of spines on its capsules, and the separation of its petals. Miss Satin has made a study of young flowers of the *quercina*-like gene type and finds that the young petals take the form of an inverted "U" by the more rapid growth of the outer side and hence the petals are never able to meet and unite at their edges as they do in normals. Neither the *quercina* disease nor the gene with similar effects causes a separation of the

calyx lobes. In another gene type, however, both corolla and calyx have separate lobes. In this case the sepals also are kept separate by an excessive growth of the outside tissue which bends the edges inward and thus prevents them from uniting.

The similarity of the end results suggests that both the virus particle and the gene bring about their effects by a similar change in the environment. Similarity of end results, however, does not prove identity of the causal agent. Similar effects in inducing gene mutations are brought about by such diverse agencies as X-rays, radium, heat and aging of seed, and several other chemicals besides colchicine have been found to induce doubling of chromosome number. It would be unwise therefore to suggest too strongly that there may be a close similarity in the nature of genes and virus particles with the difference being chiefly in the fact that genes are imprisoned in separate chromosomes and limited in their reproduction by the divisions of nuclei whereas the virus particles are unconfined and capable of unlimited multiplication. Such a possible relationship between genes and virus particles which has been suggested by others is given a measure of plausibility by the appearance of the *quercina*-like mutant type and seems worthy of further exploration.

Chemical Regulation

The success in inducing a doubling of chromosome number led us to the belief that it might be possible to find a stimulus which would reduce the chromosome number by one half. That this should be possible is indicated by the fact that the plant frequently does it. In Datura we have records of the spontaneous appearance of over 200 haploid ($1n$) plants which have arisen apparently through the development of unfertilized egg cells into viable embryos (13). Since haploids in Datura can be readily transformed to diploids by colchicine treatment, a method of securing haploids at will would be of considerable value in securing pure lines from highly heterozygous material such as species hybrids (6). The attempts by Drs. van Overbeek and Conklin to induce unfertilized egg cells to develop into embryos through the stimulus of a considerable number of chemical substances has so far been unsuccessful. From their studies (17) it has been possible, however, to show that there are several stages necessary in the stimulation of embryo formation. The first is furnished by the growth of pollen tubes which initiates development in placenta and integuments of the ovules. A second stimulus is neces-

sary for further growth of the ovary which prevents its abscission. The growth and retention of the capsule seen in parthenocarpy may be induced by injections of various auxins such as naphthaleneacetic acid. A further stimulus seems necessary for the development and differentiation of the unfertilized eggs. The naphthaleneacetic acid stimulates the inward proliferation of somatic cells lying just outside the embryo sac to form pseudoembryos in the place where the true embryos would be expected. If some stimulus could be found to induce the pseudoembryos to differentiate we should have the formation of apogamous embryos similar in origin to those in citrus forms. A seed of an orange, for example, contains several embryos but only one is sexually formed—the rest being apogamous. These latter give rise to seedlings all alike and identical in genetic constitution with the parent tree from which the embryos arose through a process of vegetative reproduction inside the embryo sac. The embryo sac appears to exercise some unknown and mysterious influence upon the growth of the seedlings which differ from young trees derived from buds or grafts of the parental type in being thorny and failing to set fruit for a dozen or more years. The juvenile characteristics of these apogamous seedlings gradually wear off but so slowly that growers cannot use them for the commercial propagation of their varieties.

There are many other growth patterns besides those involved in embryo formation which may be subject to chemical regulation (6). What are the factors which in Datura cause the terminal bud after the formation of a dozen or so leaves to stop monopodial growth of the shoot and to produce thereafter a dictotomously branched system with flowers in the center of the forks? The length of day evidently has some influence since in the summer there are many more nodes formed on the stem before forking is started than in winter. Again we are ignorant of what the factors are which cause the change from mitotic division of chromosomes without change of their number to meiosis when the chromosome number is reduced to half. There are many similar growth patterns in which chemical regulation may be involved. There is no evidence that the chromosomes themselves change in their genetic content as the organs pass through the various stages of differentiation. Rather it would appear that with this differentiation the chromosomes exert their influence upon constantly differing environments with a resulting continuing change in the product of their activity.

Such an interpretation is in accord with well known chemical re-

actions which may be quite different for a given compound when the environmental conditions are altered. Thus litmus is pink at a low *pH* but becomes blue when the *pH* is raised. A mixture of pepsin and ptyalin at a low *pH* would split proteins into more simple products such as polypeptides and amino acids but would have no effect upon starch. At a high *pH*, however, the mixture would have no effect upon proteins but would split starch into dextrose. In the two examples given there is a marked difference in the end products depending upon the *pH* of the environment.

We have given some examples of the known and possible influence of chromosomes upon growth patterns in plants and have taken examples from forms with which we were personally familiar. Perhaps our discussion can be summarized by expressing the belief that chromosomes exercise their control of growth patterns not through a single force but through continuing interaction with factors both within and without the organism.

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SPATIAL AND TEMPORAL GROWTH PATTERNS IN COLONIAL ORGANISMS

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The origin and nature of pattern may in some ways be more readily studied in lower animal groups, although the phyletic diversity responsible for this makes a comprehensive picture the more difficult to obtain. The occurrence of colonial organisms in many of these phyla extends the mystery and puts certain problems in sharper relief.

Most of the problems of development are inherent in the study of pattern at any level. Conklin (1929) defined these as being threefold: when and how do progressive differentiations arise, how are coordination, orientation, and regulation brought about, and how can one explain the teleological character of development where the end seems to be in view from the beginning. According to Sinnott (1937) the main problem is the "fundamental paradox that protoplasm, itself liquid, formless, and flowing builds those formed and coördinated structures of cell, organ, and body in which it is housed." Later (1939) he makes this criticism "the repeated failure of these various attempts to solve the problem of organized development by cutting up the individual into smaller and smaller unitary elements breeds the suspicion that here again, as in many other scientific problems we have been confusing analysis with solution," and goes on with the accusation that "the more tenderminded . . . have needed little encouragement to run after the strange gods of mysticism and metaphysics and have set up in their midst the golden calf of entelechy. Even those who remain in the ranks of the orthodox speak the unfamiliar language of holism, organicism, metabolic gradients, allometry, organizers, morphogenetic fields, gestalten and other words outlandish in the ear of analytical biology and which for the most part are merely the terminology of enlightened ignorance." To discuss organization and avoid these terms will be a novel task.

A.

All organisms exhibit organization and pattern, and colonial organisms more emphatically than others since there is not only organization on the cellular level but whole or partial organisms are themselves

coördinated and subordinated into a single entity. Metamerism should perhaps be included in this phenomenon.

One of the most puzzling, best known, and widely ignored phenomena of development and form is that of hydrozoan coelenterates, often discussed by the misleading descriptive phrase "alternation of generations." If the current concept of an organism as existing in a space-time continuum is adopted the phrase loses all meaning and one sees a single being of colonial type exhibiting in its most challenging form the phenomenon of polymorphism. Apart from specific differences in hydranth and medusoid structure, several questions immediately come to mind. How can such different patterns as hydranth and medusa be expressed by developing masses with the same genom? What determines whether the one or the other shall develop? Why is there such variability in the relative positions at which medusae develop? Gross and Huxley (1933) used a phrase, "alternative differentiation," purely descriptive, that may be usefully employed in grouping together phenomena such as polymorphic developments. They used it however in connection with regeneration of parapodia in the polychaete, Sabella. In this form thoracic and abdominal parapodia differ greatly, and if an abdominal parapodium at any level is removed or injured it is generally replaced by one of the thoracic type. If this same worm is divided in the abdominal region, a new thorax is reorganized out of abdominal segments under the influence of a new regenerating head. Under certain conditions when the new head is removed and allowed to regenerate several times in succession, the reorganizing effect spreads farther and farther down the worm in an equivalent number of steps, as though tissue already reorganized no longer offers any resistance to the reorganizing agent, whatever that may be (Berrill and Mees, 1936). In such manner thoracic structure has been extended from the normal six or seven segments to as many as 80.

Another common occurrence is a segment single on one side, double on the other. When the reorganizing effect spreads through a region containing such a segment it usually affects an equal number of segments on each side, although this means a reorganization of but one side of the last segment affected. This suggests that the segmental organization is more significant than the extent of tissue involved.

This brings us to another family of polychaetes, the syllids, the subject of several interesting papers by Okada (1933, '34, '35, and '37). In Procerastea the asexual worm develops a sexual head at maturity always on the 14th segment. If anterior to this there is

however included a segment double on one side single on the other, the 14th segment will not be the same segment on the two sides. In this case two sexual heads are produced one behind the other corresponding to the right and left 14th segments respectively.

Syllids also exhibit head polymorphism, the sexual heads differing markedly from the asexual head and from each other. But should an immature worm be cut at the 14th segment the head that develops will be of the asexual kind.

Other syllids are equally fascinating. Myrianida forms a terminal chain of many sexual individuals all arising from a single zone of growth. In one species of Trypanosyllis collateral buds are produced by many segments to form a most peculiar picture, while in another species as many as 34 sexual stolons with heads arise more or less at one time from as many segments of the parent stock.

Among ascidians are to be found solitary forms, social aggregates resulting from budding, and true colonial organisms in which the individual ascidian zooid has become a transient unit of a larger system. Botryllus is an example of this last condition. Colonies form encrusting masses in which the members are embedded in definite patterns and are linked together by a common circulatory system. Each member has an active life of merely a week or two, each new generation arising from buds produced by the preceding one at precise locations and time in the course of bud development. Bud primordia have become a very precise part of the general organization.

Such are a few examples of the pattern developed in space and time in these compound types. They merely extend the problems of pattern inherent in the formation of any organism. The study of development of pattern has for the most part been confined to developing eggs. Before discussing the significance of the egg from this point of view, it is of some interest to compare the size of eggs with the size of minimum regenerates or reconstitutions. In the case of certain lower animals attempts have been made to determine just how small a part is capable of forming a new organism out of its contained material. The facts are sketchy but suggestive, as Table 1 shows.

Fragments smaller than those indicated fail to undergo reconstitution or regeneration to form a new individual, however simplified. The order of size obviously is the same as that of the eggs which is representative of the minimum egg size of the vast majority of oviparous marine invertebrates. In both cases we are considering protoplas-

TABLE 1

Animal	Minimum size (diameter) of pieces capable of complete reconstitution	Approximate diameter of eggs of same animal
Hydra (Coelenterata)	170 microns	180 microns
Clavelina (Tunicata)	300 microns (not compact tissue)	230 microns
Lineus (Nemertinea)	150 microns (150x40x40μ)	120 microns
Ephydatia (Porifera)	200 microns (200x200x30μ)	140 microns

mic masses, one unicellular and the other multicellular, that develop into an organized individual without recourse to material other than that initially present. There is possibly an indication here as to the reason eggs are no smaller than they generally are.

B.

As cells, eggs are always relatively large, and even the smallest such as those of certain polychaetes are not less than 60 microns in diameter. And even the simplest exhibit some form of organization. Before the onset of cleavage there is in greater or lesser degree a precocious development of an organized condition, expressed as a stratification, concentric arrangement or the formation of crescents of specialized plasmas.

It is important to determine, if possible, in what manner the early organization and behavior of the developing egg may be related to the final emergent pattern. A comparison of the development of three of the lower chordates, Amphioxus (Conklin 1933), the ascidian Styela (Conklin 1905, 1929), and the larvacean tunicate Oikopleura (Delsman 1910), is illuminating.

All three have transparent eggs of approximately the same size. All develop the basic chordate organization of notochord, dorsal tubular nerve cord, lateral tail musculature, and gill slits. In Amphioxus there is further growth and elaboration without any significant change in character. In Styela, apart from gill slits, the chordate structures are more precociously developed and are but transient, surviving in functional form only for a few hours, when a destructive metamorphosis ensues. In Oikopleura development closely resembles that of Styela, although yet more precocious, but neither metamorphosis nor marked postlarval growth occur.

In these three types, at or before fertilization, there is a flowing

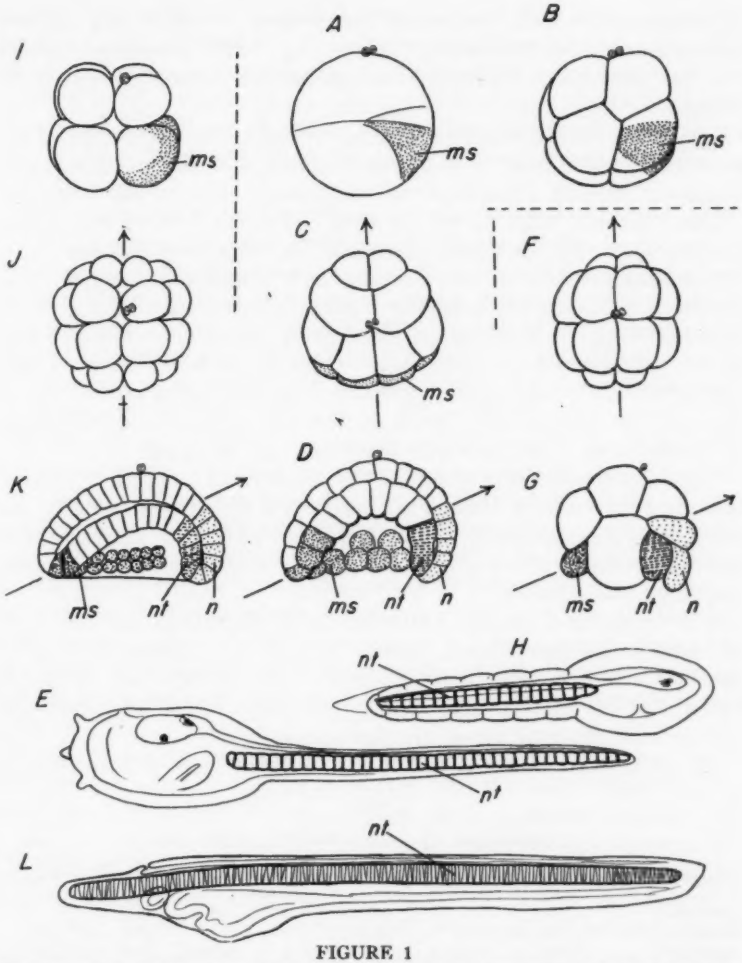

FIGURE 1

DEVELOPMENT OF THE EGGS OF STYELA, OIKOPLEURA AND AMPHIOXUS, BASED ON CONKLIN AND DELSMAN

A-E Styela, *F-H* Oikopleura, *I-L* Amphioxus. *B* and *I* show mesodermal crescent in early cleavage; *C*, *F* and *J* bilateral symmetry of cleavage pattern; *D*, *G* and *K* mid-gastrulation stage showing axis and distribution of mesodermal and chordal crescental material; *E*, *H* and *L* swimming larvae with approximately 40, 20, and 300 notochord cells respectively; *ms*, mesodermal crescent; *n*, neural plate; *nt*, notochord.

of cytoplasm, so that specialized zones form posterior and anterior crescents imposing bilaterality on the egg. This precedes cleavage and the subsequent bilateral cleavage pattern must accordingly be derivative.

Crescentic zoning, bilateral cleavage pattern, the association of the anterior crescent with chorda-neural tissue and the posterior with mesoderm indicate a fundamental relationship between the three.

The crescents must include in their substance histogenetic agents in some form. At the same time while the crescental plasmas represent histogenetically the essential chordate tissues and are truly presumptive, they are not in proper mutual relationship. This is established only as the result of gastrulation, which accomplishment may be the main purpose of gastrulation even as the formation of a two-layered vesicle. Only as the gastrulation process nears completion do the crescents come to be in juxtaposition and make possible the orderly development of the chordate swimming tail and trunk.

The histogenetic action within the chordal and mesodermal crescents is more rapid in Oikopleura and Styela than in Amphioxus, and more so in Oikopleura than in Styela. In each case subdivision or cleavage of these zones is gradually inhibited as histological differentiation becomes visible.

Most striking is the close correlation of the rate of differentiation of chordal and mesodermal tissues in each case, the correlation between this and the time of inception (in terms of cleavage) of gastrulation, and the marked and unitary manner in which these processes and correlations vary among the three types.

It can be seen from the following table that in Amphioxus gastru-

TABLE 2

Animal	Gastrulation		Notochord		Tail muscle		Egg
	Approx. cell no.	Cleavage	Approx. cell no.	Divisions	Approx. cell no.	Divisions	Diameter (mm)
Oikopleura	(38)	5-6	(20)	4-5	(20)	4-5	0.09
Styela	(76)	6-7	(40)	5-6	(36)	5-6	0.13
Amphioxus	(780)	9-10	(330)	8-9	(400)	8-9	0.12
Petromyzon	(2,200)	11	(500)	9	—	—	1.00
Triturus	(16,000)	14	(1,200)	11-12	—	—	2.60

lation occurs between the 9th and 10th cleavage, in Styela (and all other ascidians) between the 6th and 7th cleavage, and in Oikopleura between the 5th and 6th. In Amphioxus the number of chordal and lateral muscle cells formed from the egg is of the order of 300, repre-

senting between 8 and 9 subdivisions of the original zones. In Styela the number of chordal and of tail muscle cells is in each case about 40, representing between 5 and 6 subdivisions. In Oikopleura the number is 20, or between 4 and 5 subdivisions. Inasmuch as the regions subdivided are about the same volume in the three cases, the size of the cells produced in each case is inversely proportional to their number.

The conclusion drawn from these facts is that the precocious histogenetic processes and the process culminating in the visible movements of gastrulation represent a single coördinated mechanism initiated in these eggs at or before fertilization and responsible primarily and possibly exclusively for the development of the essentials of chordate structure and organization. The rate of action of this mechanism varies as a whole, and differences may be due to differences in initial concentrations of reacting substances.

In Amphioxus the structure thus developed persists as the basis for that of the adult. In Oikopleura the equivalent structure also persists into the sexually mature form, but the number and size of the chordal and muscle cells remain unchanging in contrast to the anterior trunk region, indicating retention of larval structure. But in Styela, and in all other ascidians, to which Oikopleura is related, all this elaborate construction is broken down at metamorphosis, and the development of the embryo up to the time of tadpole activity seems to have little connection with the adult organization that finally appears. In these there seems to be a residual development proceeding at a much slower pace.

If such a duality exists in Amphioxus and others with typical direct development it must be of the form of a mutual reinforcement making distinction impossible. But in ascidians there is contrast instead and it is clear that the mechanisms evident in early egg development are the basis of the so-called "historical" aspect of development emphasized by Bertalanffy (1933) and others.

One may define an egg as a relatively enormous cell possessing special histogenetic regions organized as crescents or gradients that become reorganized by gastrulation and expressing whatever historical aspect development may possess. In solitary or colonial ascidians the final organism develops from regions of the egg not precociously histogenetic nor essentially rearranged during gastrulation. This part of the development is in many ways similar to the development of buds.

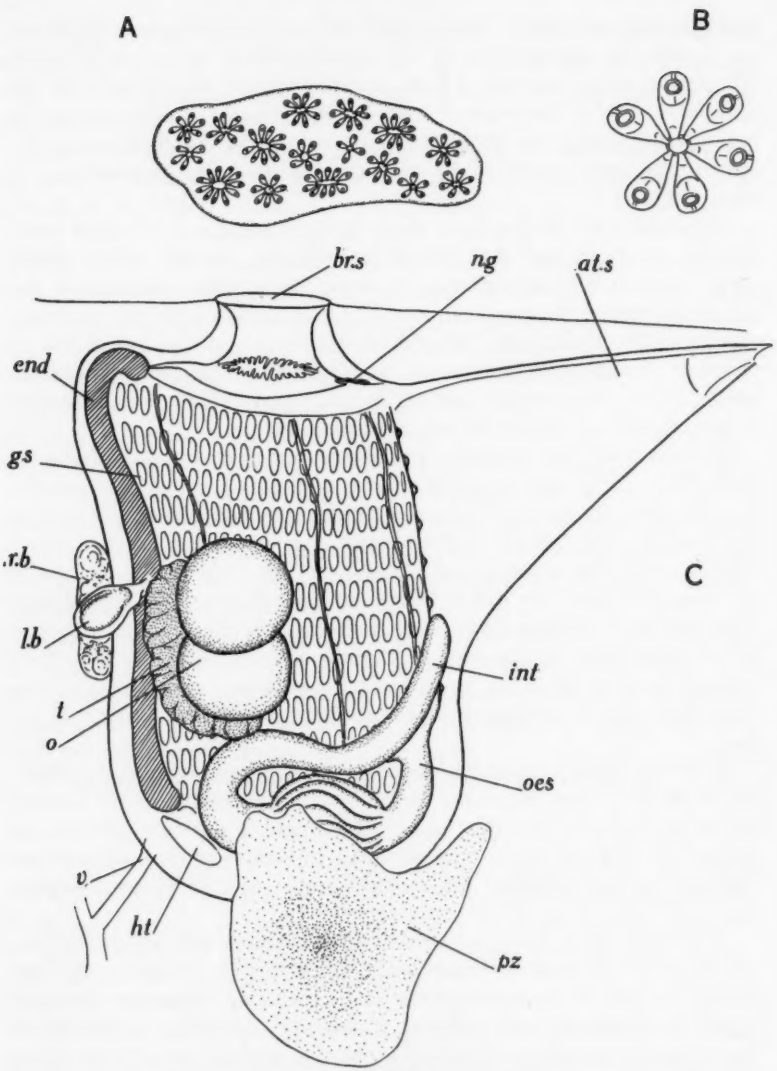

FIGURE 2

ORGANIZATION OF BOTRYLLUS

A, whole colony of star systems. *B*, a single star system with central common cloacal chamber. *C*, a mature zooid bearing both mature ova and developing buds and showing remnant of parental zooid of preceding generation; *at.s*, atrial siphon; *br.s*, branchial siphon; *end*, endostyle; *g.s*, gill slit; *ht*, heart; *int*, intestine; *l.b*, left bud; *o*, ovum; *oes*, oesophagus; *pz*, autolyzed parental zooid; *r.b*, right bud; *t*, testis; *v*, vascular stolon connecting zooid with vessels of colony.

C.

We come finally to the main point of this discourse. This is that the basic and most direct, simple type of development is to be found not in eggs with their highly specialized mechanisms, but in the meristematic development of buds or units of colonial organisms. While this type of development is undoubtedly represented in the development of eggs, it is at the same time more or less inextricably interwoven with precocities, interpolations, and retentions.

In many instances of ascidian budding, buds are relatively large parts of the parental organism and reconstitution is typical regeneration of missing structure. In others the primordium that develops is so small and histologically so simple that the development is a complete one. Such buds consist of two layers of tissue, an outer one derived from the parental epidermis and giving rise to all the epidermis, though nothing else, of the new organism, and an inner layer of formative cells that produce all the remaining tissues and structures. To take three examples, the formative cells in buds of Botryllus arise from atrial epithelium, in Distaplia from epicardial epithelium, while in Perophora they come from the mesenchymatous septum of the stolon. In other words they may originate from tissues originally ectodermal, endodermal, or mesodermal, but in every case such tissues have remained simple limiting membranes of which the cells presumably have undergone no histological or cytological specialization. Such cells may be regarded as protoplasmic units of no less potency than eggs and with nothing of their elaborate specialization.

In each of these genera one of the earliest stages of the bud consists of a sphere of two concentric layers of tissue, the outer being epidermal, the inner composed of the formative cells.

Fundamentally the subsequent development is also alike. In no case is there any semblance to the chordate organization, or historical aspect of development so dominant in the embryo. Moreover the whole progression of development can fairly accurately be described as a sheet of tissue expanding three-dimensionally from a hollow sphere which almost entirely by foldings, in-pushings and out-pushings becomes the complexity of compartments, structures, organs, and systems that is the functional organism.

For the rest the process in Botryllus will be described in some detail. It is to be noted above all for its simple directness. If the foreshadowing of the end in the beginning is evident in the development of eggs, it dominates here.

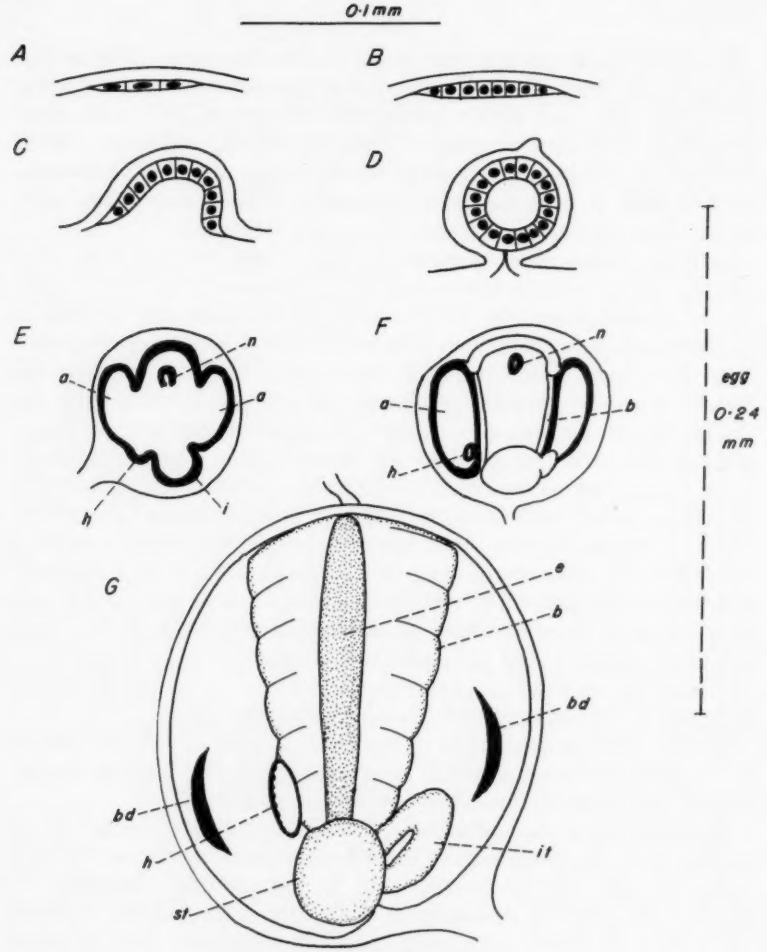

FIGURE 3

EARLY DEVELOPMENT OF BOTRYLLUS BUD

A, section of disc in earliest visible stage; *B*, disc at maximum state; *C*, transformation of disc; *D*, closed sphere stage; *E*, subdivision of sphere into primary regions; *F*, first establishment of organization units; *G*, critical stage in which stigmata about to perforate and bud discs of next generation already established; *a*, atrial chambers; *b*, branchial wall; *bd*, bud disc; *e*, endostyle; *h*, heart; *i*, intestinal outgrowth; *it*, intestine; *n*, neural tube; *st*, stomach.

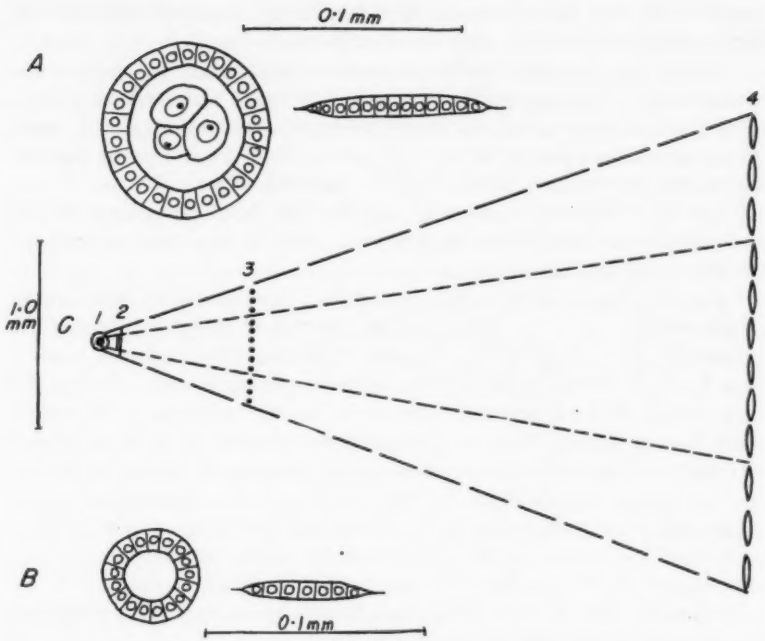

FIGURE 4

DIMENSIONS OF WHOLE, AND NUMBER OF GILL SLITS DEVELOPED FROM BUD DISCS OF LARGE AND SMALL MAXIMUM SIZE

A, disc and closed sphere stage of large size; *B*, disc and closed sphere stage of small size; *C*, size and development of gill slits; C^1, the two spheres at 1/10 scale; C^2, relative length of presumptive branchial wall; C^3, relative length of stage shown in Figure 3*G* and number of rows of perforating stigmata; C^4, relative length of emergent zooids when fully developed, showing size and relative number of rows of gill slits in each.

Starting with the closed sphere stage and considering mainly the inner formative layer, two folds divide it into a median and two lateral divisions thereby establishing the right and left atrial cavities and the central pharyngeal sac, i.e., the primary spatial divisions of the organism-to-be. Then evaginations from the central chamber establish the intestinal loop posteriorly, ventrally the heart and dorsally the nerve-tube. Somewhat later the walls of the central sac become perforated simultaneously by rows of minute stigmata or prospective gill slits. At exactly this stage, neither sooner nor later, the bud primordia of the next generation appear as slightly thickened discs in the outer wall of the right and left atrial cavities. Before this the ex-

ternal form has been more or less completely acquired although the dimensions are less than one sixth of the final size.

Further development consists mainly of expansion and histological elaboration. Throughout the whole development the epidermal layer plays a relatively minor rôle, conforming to or developing the shape of the whole and producing as a structure of its own only the stolonic outgrowth that unites the bud to the colonial system of vessels.

The fact that it is possible to describe the developmental processes so briefly even in outline indicates to some extent the monumental directness of the phenomenon.

Several points merit further emphasis. One is the rigorous correlation of the features characterizing any given stage, so that if the developmental stage of one feature is known the stage as a whole can be accurately defined. The separation of the heart evagination indicates a definite degree of development of, for example, the digestive loop or neural complex. In the development of pattern there is accordingly an invariable relationship at a given moment, with certain exceptions to be discussed later. There is no shifting in the first appearance of a character to an earlier or later stage. Such constant relationships, while obvious and possibly universal, gain rather than lose significance by virtue of their general occurrence.

Another point is the general relationship of a stage of development to its spatial dimensions.

The sphere stage already mentioned is less than one tenth of a millimeter in diameter, consisting of the order of 100 cells. It is no more than a tenth of a millimeter long when the atrial divisions, neural, heart and intestinal evaginations are all discernible, and the characteristic shape of the organism is already emerging. It is hard to conceive how the gross architecture of the organism could be expressed in a smaller entity or by significantly fewer cells.

One gains the impression that in some manner there is a pressure or thrust of the pattern against the limitation of size, or perhaps better a given size permits a certain degree of expression of the pattern. There is no doubt that size itself has a permissive quality, at least in a negative sense, since an inadequate material basis is necessarily incompatible with the expression of a dependent character.

Two possibilities exist. The first appearance of a given feature may be dependent upon the size attained at the moment of appearance or upon the size of the presumptive area at some earlier stage.

In young Botryllus colonies constituent zooids are relatively small

and bear correspondingly small bud primordia. In sexually mature colonies they are both much larger. These are not the same zooids but are separated by many generations within the colony.

The first precise stage is a disc. The disc progressively becomes more convex and finally forms the closed sphere stage. The disc at the beginning of its transformation and the sphere when just completed are two sharply definable stages. Small discs produced by small zooids become small spheres, large discs large spheres. If then the visible expression of pattern or structure is dependent on absolute size or cell number the size of disc and sphere should be without effect. But it so happens that the final size and the size of any developmental stage vary directly with the size of these earliest stages. Spheres 0.080, 0.045, and 0.035 mm. diameter give rise to zooids 2.6, 1.5, and 1.1 mm. long respectively, equivalent in each case to a linear expansion of about 30 times. If, as this suggests, the size of presumptive areas first laid down determines the size of the areas and associated structure developing from them, it should be obvious in the case of multiple structures such as the gill stigmata.

Functioning gill slits are approximately all of the same size whatever the size of the individual zooid, each being about 0.15 mm. long and consisting of about 50 cells in optical section. When first distinguishable in the developing bud each is of the form of a rosette of six cells, in optical section, with a minute central perforation. At all stages the stigmata are arranged in regular rows.

While the mode of development and final size of a gill slit is the same in all zooids, the number of rows, number per row, and consequently the total number of gill slits varies directly with the final size of the whole organism. To take two examples, the zooids 1.5 and 2.6 mm. final length, the first has 6 rows of 12, the other 10 rows of 22. The total numbers are 72 and 220, or a ratio of just over 1:3. These numbers are directly proportional to the square of the linear dimensions which vary as 1:3.2.

The same dimensional relationship holds however for early as well as late stages. The relative areas of the branchial walls at the time of first perforation and the relative areas of the same walls when they first come into existence by the partition of the sphere are equally of the same ratio of about 1:3.

In consequence of these relationships and of the fact that the full complement of gill slits appears as minute perforations at one moment in an early stage, it is clear that the number is a function of the

dimensions of the presumptive area. These in turn are a function of the size of the sphere and this is equally related to the size of the disc at its maximum state. Whatever the maximum disc size and constituent cell number may be, there is an increase of about 50 per cent in number of cells by the time the sphere stage has been formed. The final length attained by the zooid is approximately 35 times the diameter of the maximal disc. The area of the branchial wall first formed expands about 9 times before the stage is reached at which stigmata perforation occurs, and the wall with its developing stigmata expands a further 15 or 16 times before expansion ceases and the gill slits function. These are constant relationships independent of absolute sizes other than that attained by the disc stage as such.

We have then this general picture of a primary disc stage of a certain critical size, expanding and by manifold folding in three dimensions transforming directly into a complex organism of the order of a thousand times its original mass, the size of the disc at its maximum state functioning as a determinant of the final absolute size, the size of any intermediate stage or any part thereof, and of the number of multiple organs the organism may possess.

Such a conception of the significance of initial size of primordia has been put forward by others in connection with very different material. Sinnott, as early as 1921, showed that the size of the meristem in plants determines the size of any given organ developing from it. Similarly, investigating hybrid vigor in plants, Ashby (1937) and Luckwill (1939) concluded that increase in size is already determined in the embryo, and heterosis is due to the maintenance, on a logarithmic scale, of an initial advantage in primordial size without any augmentation of the metabolic efficiency or the relative rate of growth subsequent to germination. MacArthur and Butler (1938) came to somewhat similar conclusions. While from the study of the development of Bar eye in Drosophila, Steinberg (1941) concludes that the differences in eye facet number between homozygous bar, heterozygous bar and wild type are determined by differences in original imaginal disc size, and that the capital of cells involved in initial formation is different in various mutants and the growth rate is the same in all throughout development.

Before discussing further the nature of these disc-like entities there are certain other aspects of their later development that are enlightening, or perhaps one should say, confounding.

Sinnott has recently (1939) described one of these in the follow-

ing way, "the mass of material being poured into the young fruit is growing at a constant rate regardless of whether this mass is being cut up into new cells rapidly, slowly or not at all . . . (and) the unity of behavior, and thus presumably the unity of organization, inheres in the whole and not in its elements." Waddington (1940), commenting on this, suggests that cells are not the right units in which to explain growth. He does not say what the correct units may be, and in fact it is possible that there is no such thing as a correct unit, at least above the chemical level.

What holds for the growing fruit applies equally to the bud development of Botryllus.

In the description so far given nothing has been said of the gonads, which in this form are bilateral and hermaphrodite. At the end of bud development the ova are fully grown and mature and in volume equivalent to about a third of the total organism. Ova and other cells are segregated during the closing phase of sphere formation, that is, most precociously. Once segregated from the sphere wall they continue to grow without any further division.

In contrast the rest of the developing bud undoubtedly consists of small rapidly dividing cells. The latest of all to cease division and to differentiate appear to be the spermatogonia. Between the two extremes lie such tissue as muscle in which there is rapid division and minimum cell size during early stages, cessation of division followed by extensive cell enlargement during later stages.

Yet the growth for the whole is unitary. The growth rate of the non-dividing ova is virtually the same as that of the rapidly dividing cells constituting the rest of the growing organism. The rate of increase is the same whether the growing substance is being subdivided into cells or not. Everything comes harmoniously and finally into functional gear at one and the same time. The cellular state may be an essential condition of development and organization but it is decidedly not its basis. One is driven to accept Sinnott's conclusion that the unit in development is the entire cellular mass, although as he says it is certainly not helpful to talk of it as the organism as a whole.

There is another aspect of this development which is possibly the most interesting. This concerns the limiting control effected by the cell number and absolute size of a given developmental stage. The cell number of a particular stage is a function of the size or cell number of the preceding maximal disc stage. Thus the sphere stage may show

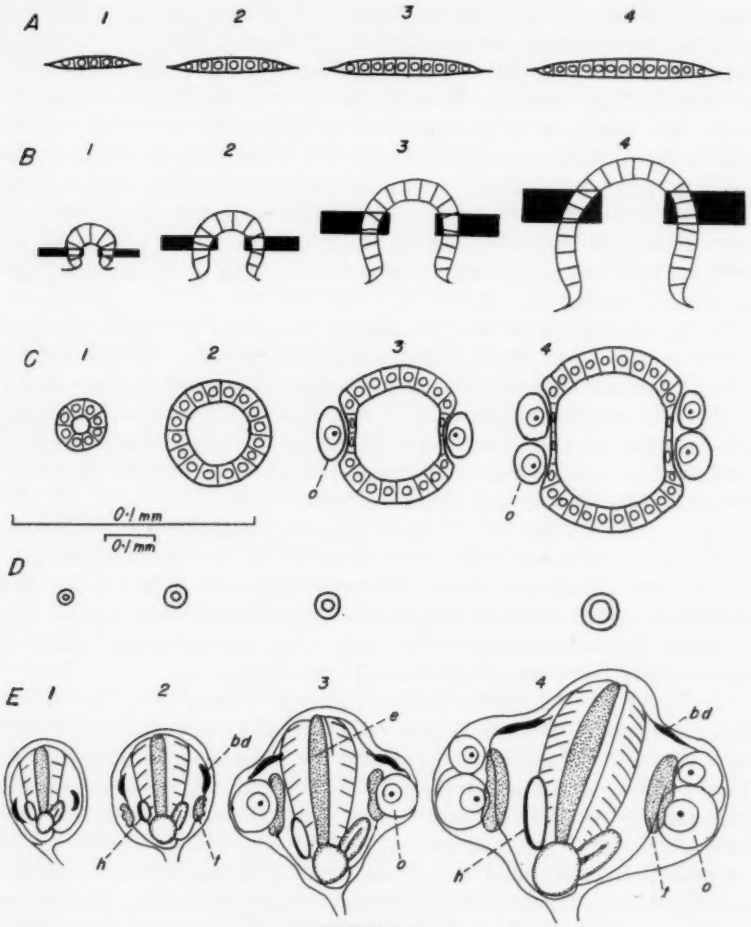

FIGURE 5

DISC SIZE AND GONAD FORMATION

A, discs of maximum sizes 1-4; *B*, closing spheres developing from discs 1-4, with extent of presumptive gonad area indicated in each case by black-out; *C*, continuation of same series 1-4 to closed sphere stage, showing failure of ova to segregate in 1 and 2, single and double segregation in 3 and 4 respectively corresponding to number of cells included in presumptive areas in preceding stage; *D*, sphere stages 1-4 reduced to 1/5 scale; *E*, continuation of series 1-4 on reduced scale as far as bud disc stigmata stage, showing subsequent development of gonads in the four sizes; *bd*, bud disc; *e*, endostyle; *h*, heart; *o*, ovum; *t*, testis.

from 9 to 25 cells in optical section, representing totals of from 33 to 210. Ova are segregated from the wall even before the sphere is closed and when even fewer cells are present, at approximately the hemispherical phase. Obviously segregation or extrusion from the wall can only take place in the form of complete and distinct cells. It is interesting to determine the approximate number of cells in the presumptive gonad area at the time proper for extrusion in pre-sphere stages derived from discs of different size. When extrusion has just occurred and before the cells extruded have grown beyond the size of those remaining in the wall, it is possible to tell with fair accuracy the area they occupied immediately before.

In an open sphere stage of about 18 cells in optical section two cells in each wall represent presumptive ova. Altogether three or four cells are extruded as ova but in optical section only one or two are included. Spheres of this type producing three or four ova on each side however are not the smallest but the largest, associated with the production of large sexually mature zooids.

If the equivalent stage associated with segregation of ova is reduced to two-thirds the size of that just considered, the presumptive area instead of containing three or four extrusible cells will contain only one complete cell. The rest of the territory will extend over merely parts of neighboring cells, and only the one cell can be extruded. Thus in open sphere stages of about 12 cells in optical section usually but one ovum is produced on each side. In equivalent stages of smaller dimensions and cell number the presumptive area at the critical moment for extrusion would not contain even one complete cell. In such cases no ova are extruded whatever. Consequently the smaller buds typical of early generations in a colony are either asexual or without full gonad complement.

Accordingly while cells do not appear to be directors of events the response to direction is conditioned by the number of cells or degree of cellulation in the region concerned. In the case just described segregation was inhibited by inadequate subdivision.

It is equally notable that what should be formed at a given stage and is not, is not formed at any subsequent stage. Ova not formed during the sphere phase do not appear later.

Mature ova however are but one component of the gonad. They are merely the first to segregate. They are followed by male cells and by vestigial ova that never grow to maturity, and finally by male

cells alone. Each phase of gonad segregation occurs at a precise stage of the whole, neither sooner nor later.

It is possible that while subdivision of the presumptive area may be inadequate for extrusion of the prospective mature ova of the first segregation phase, adequate cell numbers may exist for the segregation of other components at a somewhat later stage. It is a fact that in zooids of later generations than the early asexual ones but not so late as those containing mature ova, gonads are represented by imperfectly formed testes alone or accompanied by vestigial ova.

When no gonad material whatever is segregated from the lateral walls of the sphere, the tissue later becomes mesenchyme, muscle tissue, atrial epithelium and even part of the bud disc of the next generation. This reminds one of Lillies' theory of dichotomous segregation (1929). There is clearly an opportunity that must be taken at the proper moment or be forever lost. One should also remember in connection with the presence or absence of reproductive organs that in plants differences in the shape of the meristematic cone are associated with the production of primordia of vegetative and of reproductive organs respectively (Sinnott, 1938). While not directly comparable, Stern (1940) has recently discussed the imaginal discs of Drosophila with reference to the number of cells entering into their constitution and the significance of subanlagen consisting of as few as two cells. Obviously any reduction in size at a critical stage would here also result in striking consequences.

So far we have traced pattern in the sense of determination of size relationships and some material segregation back to a stage close to the disc at the time of its transformation into a sphere. It is evident that by the time the disc has reached its maximum size something momentous has already occurred with regard to the laying down of the pattern.

The disc can be recognized when consisting of no more than six or eight cells. How small this is can be realized from the fact that the egg of Botryllus, itself a quarter millimeter in diameter, has a volume of the order of 12,000 times as large.

The disc may grow until it reaches a maximum constitution of from 20 to as many as 160 cells, a difference equivalent however to merely three divisions. What is responsible for such differences is not known. At some stage in this expansion the imprint of pattern, whatever its basis, becomes stabilized and limited.

There is one phenomenon which is definitely related to conditions

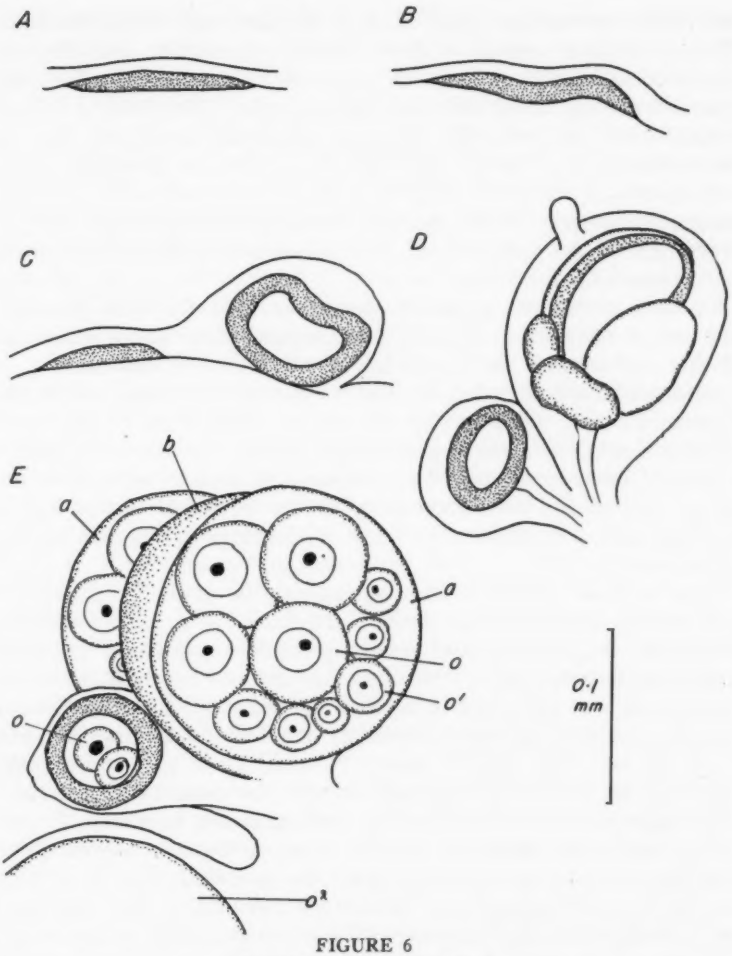

FIGURE 6

FORMATION OF DOUBLE BUDS

A-D, from early small, non-sexual zooid generation; *E*, from large fully sexual zooid of late generation. *A*, maximum disc; *B*, subdivision of disc; *C*, separate development of large and small constituents; *D*, later development of same; *E*, large and small buds derived from single disc; *a*, atrial chamber; *b*, branchial chamber; *o*, prospective mature ovum; o^1, prospective vestigeal ovum; o^2, maturing ovum of parental zooid bearing the buds.

within the growing disc itself. This is the occurrence of double buds. These grow from what is at first a single primordium, and they are associated with the small asexual bud generations and with the large sexual forms, but never with intermediate types. They can in fact be formed from large or small discs but apparently not from those of intermediate size. The determining factor is not size alone but shape.

Double buds arise from discs that are pear-shaped rather than circular. The anterior wider part gives rise to the larger bud, the narrower part to the smaller bud. Transformation to form two spheres takes place simultaneously.

In early generations no gonads are formed and the whole presumptive area is left intact. In fully mature generations at the other end of the scale gonads are completely formed without encroaching on the presumptive bud area. In between gonads are segregated to the maximum extent permitted by the state of cellulation of their presumptive area, to form reproductive organs imperfect in variable degree. Under these last circumstances the anterior margin of the gonad presumptive area encroaches on the posterior narrow end of the bud area and inhibits the later development of the smaller bud of the pair.

Two further conclusions may accordingly be made. The whole of a formative area is not necessarily incorporated as a single developing entity but the limits of the pattern being laid down may be determined at least in part by geometrical factors. Secondly when the margins of two presumptive areas of organs appearing at different periods overlap, response is made by the first prospective organ to reach an adequate cellular basis. It seems clear that the stimulus to segregate as gonad in no way impairs the capacity of the tissue to respond in other ways if the first demand cannot be met.

It remains undetermined at what stage earlier than the maximum disc the pattern is represented in any decisive state.

It is hard to conceive or visualize pattern in an invisible form, yet it is equally difficult to avoid the conclusion that in some representative or symbolic form the pattern of the organism has its expression in the disc in spite of its minute dimensions. It is evident that it cannot be a product of intercellular relationships since the constituent cells are too few and their number too variable. Everything points to a unitary system.

What develops is a growing interacting mass of cytoplasm and nuclear constituents, the organization into cells and nuclei being con-

ditioning rather than basic factors. It is the interacting system that is significant, not one or other of the components. So much has been said of the rôle of genes that one is tempted to define them as statistically significant little devils collectively equivalent to one entelechy. Harrison (1937) has already warned us of the dangers implicit in the "wanderlust" of geneticists and the hindrance the gene theory might become in directing our attention solely to the genom, when in fact all processes of development are actually effected by the cytoplasm. Stern (1936), as a representative of the invading geneticists, avoids both the ditch of genom and of plasmon, to quote, "the basis of this whole (ontogeny) is the *combination* of plasma and genes. Genes act in their plasmatic surroundings in all stages of ontogeny . . . (and) . . . the timing of specific effects of genes cannot be regarded a property of the gene itself but must be looked for in the interaction of the whole developing system."

It is well to remember too that cell delineation generally is thought to be without much significance, the only discrete elements being the nuclei, each with its protoplasmic territory (Weiss, 1940). In the blue-green algae, the Cyanophyceae, nuclear material is itself diffuse. While in the Phycomycetes organized form is maintained in spite of a general circulation of protoplasm.

Whatever the system is in reality there is no doubt it exhibits the principle defined by Bertalanffy and Woodger as the "tendency towards maximal organization." It is interesting to conjecture what happens when the maximal organization is finally reached or has been fully expressed, and one might say was on the verge of over-expansion.

In Botryllus and many other ascidians when a zooid has fully developed, produced its eggs and sperm, sheltered its embryos and grown a little, it disintegrates into its constituent cells and subsequently undergoes autolysis. It falls to pieces and dies. Neither the demands of new buds, state of its food supply, nor physical factors such as rising or falling temperature can be held responsible. The phenomenon is undoubtedly inherent.

It is as though growth or expansion having reached a maximum can proceed only into incoherence and death. This is a vague suggestion, like others in this report, and much has been included that might have been better formulated. Perhaps the responsibility should be shared by Planck whose fortifying words have already been relayed to Embryologists by Harrison that "we must never forget that

ideas devoid of a clear meaning frequently give the strongest impulse to the further development of science."

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ALLOMETRIC GROWTH: THE ONTOGENETIC AND PHYLOGENETIC SIGNIFICANCE OF DIFFERENTIAL RATES OF GROWTH

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A. INTRODUCTION

Eggs are small and approximately spherical in form,—a form which represents a stable equilibrium between internal and external forces. Fertilization upsets this equilibrium and the organism undergoes a series of rapid transformations,—essentially an unstable moving equilibrium,—until finally a new relatively stable equilibrium is attained, that of the adult form and size. The adults, many thousands of times larger than the eggs from which they develop, depart widely from the spherical in form. They are, in fact, of almost every conceivable shape and form except the spherical.

In contrast to the egg, the adult body is of unequal size along the main axes defining the three planes of space. It may show one or more major body regions and be equipped here and there with different sorts of appendages, projections, and protuberances of various form and size; and these outgrowths, as well as the ingrowths and the central body itself, show smaller swellings, bumps, and bulges in inexhaustible variety. All this is, of course, accompanied by the necessary complementary constrictions, cavities, holes, and depressions. No matter how much larger in size than the egg or how much more complicated in shape, the adult organism has its substance distributed to the parts in a definite pattern that is remarkably constant for any one genetic type.

At any moment during the change from egg to adult, the organism has a certain shape and size and certain properties. The shape, the size, and the properties vary continuously and in the most subtle ways. But the shape of the organism at any time is an expression of the size and arrangement of its parts, and of course, their balanced functioning. The structural pattern at any moment is only a provisional one. The organism continues to change. It increases in size, not uniformly in all directions, but nevertheless according to a definite pattern of growth. This ontogenetic growth pattern is not so much a concrete

thing, visible to the eye, as it is a manifold of diverse processes, proceeding, in the case of normal growth, at velocities that are somehow in equilibrium and harmoniously adjusted to one another and controlled in some way primarily by the genetic constitution. To be sure, at any moment we can see the elements already established in the provisional pattern. These we measure at successive intervals to reach a knowledge of the dynamic unity of the balanced processes. A study of the size relationships of an adequate number of stages during development has shown that the continuous series of transformations and the changes in proportion mask a constant pattern of invariant relationships. In a certain sense the more the organism changes the more it remains the same. This change from the small simple-patterned egg to the many times larger adult with its characteristic complicated morphological pattern has been brought about by the simple process of unequal growth in different directions, i.e., by the general process of differential growth.

Furthermore, when adults belonging to different but related species are compared it is found that homologous elements in the structural pattern differ in size, in shape, and in relation to one another. As the genetic relationship becomes more distant, the size differences become more marked. New elements may arise, old elements may become rudimentary or disappear, or become fused with one another, with or without a change in the functional relations. These differences in the morphological pattern as we go from adult to adult in an evolutionary series likewise represents a series of transformations which vary in a systematic manner. From the present point of view, it is obvious that the differences between the adults in such a series need to be explained in terms of the differential growth of homologous parts during their respective ontogenies.

These elementary facts serve to frame a problem that still takes the measure of the capacity and ingenuity of all students of biology, namely, the physiological problem of the assumption of form; or in other words, the problem of the origin of the ontogenetic growth pattern and the further related problem of the physiological mechanism involved in its coming to completion or maturity in the adult.

A major difficulty at the very beginning is the one of finding a suitable mode of description of the growth pattern in more than qualitative terms. A method is desired which will allow a view of the invariant relations that underlie the continuous series of changes in

size and shape during embryonic development, as well as one for the somewhat similar transformations in an evolutionary sequence.

One thinks first of the method of deformed coördinates used by Thompson (1917) to describe the evolutionary changes. This method has been recently applied to the morphological changes during growth of a salamander by Richards and Riley (1937). The value and the limitations of this method have been discussed by Huxley (1932). Briefly, the deformed coördinates demonstrate in a striking way the systematic change in the pattern but one cannot see directly from such pictorial diagrams the very thing we seek, namely, a knowledge of the differential growth processes.

In an essay on the category, "Relation," Haserot (1932) draws an interesting contrast between substance-minded and relation-minded men. The substance-minded type of thinking is unquestionably the older, both in the individual and in the race. It has all the tenacity of original sin. In morphology it has given us representative particles, preformation, the transmission of acquired characters, and such morphochemical hybrids as bristle-producing, facet-producing substances, and so on. The morphologist, when substance-minded, thinks of the developmental pattern in terms of the visible structural characteristics from stage to stage. In short, he thinks in terms of a series of pictures. But when relation-minded, the morphologist recognizes that the pattern at any moment is the expression of the events which produce it, and attempts to gain a knowledge of the durations and rates, and the relative durations and relative rates of the component processes in the developmental nexus. Consequently, instead of thinking in terms of a series of pictures, the relation-minded morphologist tends to think in terms of the non-picturable. If the problem of the developmental pattern is similar to the problems of the more exact sciences, then no doubt in time a system of equations will be developed to facilitate our thinking about it.

But the immediate difficulty is not simply one of acquiring a new and unaccustomed manner of thinking. The organism itself presents certain peculiar difficulties by its mode of development. For example, at an early stage of the organism, let us use an early Amphibian gastrula, if two points are marked on its surface and the attempt made to trace them to a later stage as the organism increases in size, we should find at a later stage that these two points have themselves become by then considerable areas or masses of ill-defined shape, and we should be at a loss to recognize the original points or trace their

movements relative to each other during the changes from the early to the later stage.

Again, if we were to mark a point on the dorsal lip of the blastopore at an early gastrula stage then later we should expect to find it as part of the roof of the archenteron. If we should use a late gastrula stage, then a point at the same topographical position would be found later somewhere in the tail-bud. Without belaboring the argument, it seems clear that topographical points of early stages are lost in expanding areas that move about in a somewhat confusing fashion in regard to their spatial and temporal relations. And besides, parts visible at later stages are not even to be seen at earlier stages. Epigenetic landscapes are non-picturable. Pictures and diagrams are, of course, a great help but the unity of the total set of events and the relationships involved can be seen only with the eye of the intellect.

In the past the most usual way to study the changing size relations has been simply to determine how the indices of the various measurements change. It has frequently been pointed out that general conclusions based on such indices may be quite misleading except for those cases where the relative growth of y and x is the same, i.e., in terms of allometric growth, when $k = 1$. Although not entirely outmoded for all purposes, this method has been surpassed, if not entirely superseded in general usefulness by the relative growth function.

B. The Relative Growth Function

Thanks largely to the impetus given to its use by Huxley, a simple power function

$$y = bx^k$$

has been found to apply to a wide variety of data, but only as an empirical approximation. In straight line logarithmic form the equation becomes

$$log\ y = log\ b + k\ log\ x$$

in which x and y represent variable elements in the changing pattern, and b and k are constants.

The data in the literature which conform approximately to this equation are not by any means homogeneous. x and y sometimes refer to the parts of the same individual as it increases in size; or x and y may refer to average measurements taken at comparable developmental stages on a series of individuals of the same kind; again x and y may refer to measurements taken from individuals grouped

into size classes, on the not entirely justified assumption that size differences have a one-to-one relation with age differences; or again x and y may refer to homologous dimensions on a series of adults of the same genetic type which differ in size. These several cases all refer to ontogenetic growth. But x and y may refer to homologous measurements of adult individuals of different size which have an interest because they belong to different but related species, and so the implications are evolutionary.

In these several instances, and they do not exhaust the possibilities, the equation applies not only to the size of the parts in relation to some measure of total size but it applies also to the parts. The parts themselves are integral wholes, the elements of which have the same type of relation to one another that the parts themselves have to the whole organism. The legs in relation to the whole organism, and the segments of the legs in relation to the entire leg are illustrations of the point. Other examples are such structures as the skull in vertebrates and fruits in plants. There are of course many examples and illustrations. In brief, the organism is a regressus of patterns,—patterns within patterns, on down to the component cells themselves whose major components likewise conform to the equation as shown by its extension to the cytonuclear ratio (see Sinnott and Trombetta, 1936) in both plants and animals. Pattern as such extends even beyond the main parts of the cell and we might speculatively infer that the visible structural pattern is in some way related to the finer pattern on the colloidal and molecular levels of organization.

The relative growth equation applies very extensively to data on the visible morphological pattern. But it might be mentioned at this point that it has been found to apply also to the amounts of various chemical constituents of the animal organism (see Needham, 1934). It applies also to data on the basal metabolism of mature birds and mammals, with the mammals ranging in size from the mouse to the elephant (Brody, Procter, and Ashworth, 1934; Benedict, 1938, see especially Figure 45). Benedict, however, says (p. 172), "it is believed that the seeming similarity between the different species shown by this logarithmic chart is an artificial similarity." To one who has no acquaintance with the technical difficulties that confront the student of metabolism, this seems somewhat like discovering a uniformity in the motions of a falling apple and the moon, and then rather pessimistically conclude that it is a mere coincidence.

Now power functions are among the most common relations found

in Nature, and although the heterogonic equation has a wide scope, yet it perhaps needs to be emphasized that it is only an empirical approximation. It has proven to be a very useful tool, but one which must be used with caution when we come to its biological interpretation, especially in regard to the significance of the constants b and k.

C. The Biological Meaning of the Constants, b and k

When any two elements y and x in the same pattern conform approximately to the equation for a shorter or longer period, then on a logarithmic gird a straight line is obtained. In terms of the graph, k is the slope of the line and log b is the y-intercept; b is the value of y when $x = 1$. Having regard to the biology and not merely to the mathematics, it is desired to give meaning to the constants b and k. In regard to these constants, we leave aside the absolute magnitudes of the variables x and y. The numerical value of k is independent of the units of measurement, while that of b is not.

First then in regard to the biological meaning of k. When the time course of x and y is exponental or parabolic then k is the ratio of the relative rate of growth of y to that of x. But when the time course of x and y is sigmoid then k, while it involves the velocity constants of the respective equations, includes also other parameters. Lumer (1937) has shown, for example, that in some cases k may have the value r_1A_1/r_2A_2, (see also Glaser, 1938) where r_1 and r_2 are the velocity constants of y and x, and A_1 and A_2 are their respective upper asymptotes. Without regard to the time course of x and y, k may be considered simply as the ratio of the percentage change in y to the corresponding percentage change in x.

Since k is a ratio of the respective velocity constants or of the velocity constants and other parameters of the functions giving the time course of x and y, it does not give us directly any information of the absolute values of the constants entering into the determination. But as a ratio the value of k is usually found between 0.5 and 1.50. Perhaps it is too early, having regard to the rough character of many of the estimations of k, to attempt to determine which values are most common. An attempt to do so, however, seems to indicate that the values do not occur at random over the interval from 0.5 to 1.50. Common values below one occur in the neighborhood of 0.73, 0.82, 0.88, and 0.95. A value near unity itself is quite common. Above unity values near 1.05, 1.14, 1.22, and 1.37 are quite frequent. In comparing values of k either from the same or different patterns it

is well to remember that after all it is somewhat arbitrary which element in the pattern is chosen as x and which as y. For example, when length and width of a skull are the variables, or the length and width of a leaf or of a fruit, it is arbitrary which is selected as x and which as y in the relative growth equation. If the selection is made in one way the resulting value of k is the reciprocal of its value when the variables are selected the reverse way.

In the series of values of k mentioned above, those below unity are the reciprocals of those above unity. A reciprocal relation of this sort between values of k was pointed out by Harrison (1934) from data on heteroplastic transplants between *Amblystoma tigrinum* and *A. punctatum*. The value of k for the growth relation between a grafted *tigrinum* eye and the normal eye of the smaller *punctatum* host is 1.37; while the same relation for a grafted *punctatum* eye on the larger *tigrinum* host is 0.73. But similar reciprocal relations may also occur for certain variables during ontogenetic development when x and y retain the same meaning. Lumer and Schultz (1941), for example, find that for the thigh length/trunk length relation during prenatal growth in *Macaca mulatta* the value of k is 1.138. During postnatal growth the value for the same dimensions is 0.89, which is the reciprocal of the prenatal value. In such a case it is clear that in their growth relative to each other the elements x and y have changed places. Such reciprocal relations are known likewise in evolutionary relative growth between homologous elements in related forms. Phleger (1940), in a recent application of the relative growth equation to fossil data on the skulls of the Felidae, finds in the jaguar that when the distance from the posterior end of the glenoid cavity to the posterior end of the condyles is taken as y against the length of the skull (from anterior end of the premaxillary to the posterior end of the inion) taken as x, the value of k is 0.72. For the homologous relation in *Smilodon californicus*, the value of k is 1.38, which is the reciprocal of 0.72 the value of k for the jaguar within the limits of the standard error of k. Again, in the evolutionary pattern in the skulls of titanotheres (Hersh, 1934; data published by Osborn, 1929), the value of k for the zygomatic width/basilar length relation for the Eocene genus Palaeosyops is 1.348, which is approximately the reciprocal of 0.75, the value of k for the same relation in the Oligocene genus Menodus. Such reciprocal relations in the value of k need to be taken into consideration in making comparisons between diverse elements in the same pattern or when different patterns are compared.

Although values of k are commonly between 0.5 and 1.50 or slightly beyond, yet exceptionally high values of k also occur and these correspondingly have reciprocal values in the neighborhood of zero. These exceptionally high or low values are related to different time relations in the cycles of growth of different parts of the organism.

Within the grand period of growth of the organism there are epochs, (see also Teissier, 1937), which involve the overlapping in time, if not a distinct sequence, in the growth of some of the sub-patterns. The wren will serve as an example to show something of this sequence as the organism develops, and the effect on the values of k. There is first the cleavage pattern which, although in regulative types of eggs seems to have no particular relationship to the later pattern of growth distribution, is, nevertheless, and especially so in mosaic types of eggs, a distinct pattern in its own right. Then cleavage is followed by the formation of the definitive embryo. As the embryo increases in size there is an antero-posterior growth gradient along the main axis. Although it has not been determined for the wren, it seems certain that the embryonic growth pattern comes to maturity before hatching. During the post-hatching cycle there is a reversal of the growth gradient as estimated from the different values of k for different body segments relative to increase in total body length. The values of k follow: head length, 0.675; neck, 0.96; body (from shoulder to base of tail), 1.019 and tail, 1.055 (Huggins, 1940). This postero-anterior growth gradient is then followed by another antero-posterior gradient as shown by the pattern of feather growth. By the time the birds are ready to leave the nest on the 15th day after hatching, the interramal feathers at the very anterior end have reached 100 per cent of their adult size. In general the feathers of the anterior tracts are farthest advanced, having reached 66-100 per cent of the adult size. The gradient falls off toward the posterior end where the rectrices have attained only 30-32 per cent of the adult length (Boulton, 1927).

Now when growth of an element from a pattern beginning later in time is plotted against the growth of an element from an earlier cycle in which growth is nearly completed then the value of k in the relative growth function will be exceptionally high and b correspondingly very low. In extreme cases the value of k may be close to infinity. Such extreme cases occur regularly in plants, for example, when the length of the seed pod or fruit is taken as y against some measure of the whole plant taken as x in the relative growth equation. But less extreme cases of the same general type occur in any organism

where there is an overlapping in the sequence of the growth of subpatterns. This occurs in the wren. Mrs. Huggins (1940) in her study of relative growth shows that k has extremely high values for the feathers (coronal, primaries, rectrices). Very few of the feathers make their appearance before the 6th day, but by the 7th and 8th day (Boulton, 1927) the pattern of feather growth is well under way in all parts of the body. By the 8th day, the body length has reached about 90 per cent of its asymptotic value for the post-hatching cycle of growth of the body dimensions. Consequently, when the length of the coronal, fifth primary or the first pair of rectrices is taken against body length in the relative growth function during the post-hatching cycle, then the value of k is exceptionally high. For any of these feathers its value is about 18.0. All the feathers at the time are growing at a rate disproportionately high relative to the body. But the growth of the body in the nestling phase is nearly finished and when the feathers have finished their own proper growth later there is nothing disproportionate in the size of the feathers relative to the body, in spite of the extraordinary high value of k. Under such conditions of overlapping time relations of elements from different patterns, even a small element from a later maturing pattern will have about as high a value of k as a much larger element. The feathers mentioned above differ considerably in size: 5.2, 28.0, and 13.0 mm. for the coronal, fifth primary, and first rectrices, respectively, at the time of leaving the nest. But within the feather pattern itself, if one feather is taken as y against some other one chosen as the standard x in the relative growth function, then values of k are much lower and tend to have more common values between 0.5 and 1.50.

The head of the wren is likewise of interest here from the standpoint of the interpretation of k. The values of k for the several head dimensions against body length during the post-hatching cycle are 0.675 for the length, 0.698 for the width, and 0.671 for the depth. Relative to one another therefore these several dimensions have a value of about 1.0 for the growth constant. Consequently, there is practically no change in head form during the post-hatching cycle. Although the head is growing at a decidedly lower rate than the body, it is not disproportionately small because during the embryonic period when the form of the head was established it was growing relatively faster than the body. The effect of the higher value of k before hatching is compensated for by the smaller value of k after hatching so

that the combined effect when growth is completed is neither a disproportionately large or small head.

It seems that the bill is involved in the antero-posterior pattern of growth as established by the growth of the feathers. On logarithmic plot, the curve is concave upward which, if fitted to two straight lines, shows that the value of k for the bill against body length changes from 1.32 before the 8th day to about 2.25 after the 8th day (Huggins, 1940). Although k is rather high there is nothing disproportionate in the size of the wren's bill, again because of the adjustment in the time relations in the growth of the bill and body length.

When, however, k is large (or small depending upon how x and y are chosen) for elements or dimensions of a part within the same subpattern then disproportionate size relations may occur. An outstanding example is the length/width relation in the snake gourd. Here the value of k is about 2.2 and when growth is complete the gourd may be over 2 m. long but have a diameter of only 4 cm. (Sinnott, 1936). Among animals, a good illustration is the face length/cranium length relation for postnatal growth in the baboon (Huxley, 1932, p. 19) where k is 4.25, but the whole curve follows rather the consequences of sigmoid growth.

In evolutionary allometry also similar disproportionate relations may occur within the same subpattern. For example, in the titanotheres the earliest representative of the genus Brontotherium had a cephalic index (zygomatic width/basilar length of skull) of 0.65 or less. It may have been as low as 0.53 depending upon the systematic position of an aberrant specimen, but as the genus increased in size during Oligocene times the index progressively changed until the last Brontotherium had a skull actually wider than it was long. The change was from an extreme dolichocephalic to an extreme brachycephalic skull. The data show that the value of k was 2.5 for this genus throughout the whole extent of the Oligocene (Hersh, 1934).

While disproportionate size relation in the adult may be a consequence of the continuation of the same type of differential growth in both ontogenetic and evolutionary increase in size, it might also be pointed out that conversely the same adult proportions between different species may sometimes be a result of a difference in the course of relative growth. A recent interesting example of this is seen in *Macaca philippinensis* and *M. sinicus* (Lumer and Schultz, 1941), which have about the same value of the tail-trunk index in the adult. During the postnatal period the relative growth differs markedly in the two species.

The value of k is 0.767 in the former and 1.001 in the latter species. The values of log b also differ, being 0.8395 and 0.2461, respectively.

In describing the course of differential growth by the allometric equation, it is clear that no disproportion in the pattern necessarily results from high values of k because these are commonly an indication of overlapping in the growth sequence of subpatterns. When there is a distinct sequence in the growth of x and y then k may have the extreme values, ∞ or 0, without any disproportionate size relations.

But within the same subpattern values of k are commonly between 0.5 and 1.50. When they exceed these values disproportionate size relations are likely to occur, and as growth is continued to larger and larger absolute sizes the disproportion soon becomes extreme, as in the snake gourd. When k departs from unity it is clear that changes in form occur with every increase in the absolute size of x and y. A typical example is seen in the changes of shape of gourds (Sinnott, Figures 2 and 3, 1936) where k for the length/width relation is about 0.8 for the bottle gourd, Lagenaria.

In comparing relative growth relations, the constant b needs to be considered as well as k. If we compare only the k's and disregard the b's, we lose sight of certain differences necessary to a more complete comparison. It was pointed out above that the numerical values of the growth constant k are independent of the units of measurement, but this is not true of the constant b, the initial growth index. Since it is the value of y when $x=1$, its numerical value does obviously depend upon the unit of measurement. It has another serious defect. Since conventional units of measurement are necessarily so chosen as to be smaller than the size of the object measured, it follows that the numerical value of b usually represents, in terms of the graph, an extrapolation to the left of the range of data, provided, of course, that the original units are retained in the calculations. The farther the extrapolation to the left the greater, in almost all cases, is the apparent magnification of the differences in b when the curves which enter the comparison have different values of k.

Although it has these two more or less serious defects, yet it is true nevertheless that b is required in the equation to give an approximate description of the data. Its biological interpretation must certainly be approached with caution and with due regard to the peculiarities of the particular case. The main point can be brought out by considering the relation between b and k for any set of curves that are allometrically homologous.

It has been found for a number of such cases (for references see Lumer, Anderson, and Hersh; *Amer. Nat.*, in press), that as k increases b decreases according to the simple exponential relation

$$b = Be^{-rk}$$

For any such case the original family of curves intersect at a common point (Lumer, 1936). But since the numerical value of b depends upon the unit of measurement, the unit can be so chosen that the relationship may be an increasing or a decreasing exponential function. Furthermore, if the unit used is adjusted so that the point where $x=1$ is the point of intersection of the family of allometric curves, then there is but one value of b for all the values of k given by the data (for algebraic explanation and illustrative figures, see Lumer, Anderson, and Hersh; *Amer. Nat.*, in press). In other words, for the same set of curves b may vary inversely or directly with k, or all the curves may have the same value of b depending upon the size of the unit of measurement. While under these circumstances the meaning of b may be somewhat elusive, it is obvious that the actual relationship between y and x does not depend upon the unit of measurement employed.

The point of intersection means in biological terms that at this value of x and y the individuals or the groups supplying the data are most nearly alike in their proportions, no matter what the unit of measurement employed. Consequently when the point of intersection lies at the upper range of the data, there is a convergence and when it occurs at the lower range of the data then there is a divergence of the individuals or groups supplying the data. It is obvious that such relationships can not be known merely from an examination of k for any set of curves. In any actual case where the k's differ but the b's have the same value, it means merely that the unit happens to be so adjusted as to fall at the point of intersection of the curves.

For comparing relative growth relations in different forms or groups we need to take into consideration not only k but also b (or log b). But since the value of log b represents a definite point on the graph, a direct comparison may be misleading, for where b is an extrapolation its numerical value has no direct biological meaning. An example where b is not an extrapolation, but can be given a distinct meaning directly in terms of the data is of interest in itself and can at the same time serve to indicate a possible way to adjust the value of b in a rational manner to avoid the hazards of extrapolation.

Such an example is contributed by Anderson and Busch (1941),

in their study of allometry in normal and regenerating antennal segments in *Daphnia*. At the first instar they amputated the dorsal ramus of the antenna at different levels of the first segment at a body length of nearly 1 mm. The data show that growth in length of the regenerating segment relative to subsequent increase in body length conforms to the relative growth equation, and so b in the equation gives directly the level of injury, i.e., the approximate size of the stump at the beginning of regeneratory growth. The relative growth curves for the five levels of injury and for the normal unoperated animals tend to converge toward a point, the calculated value of which is y, 0.465; x, 4.9 mm. The latter value represents the maximum body length for the normal *Daphnia magna*. Regeneration of the antennal stump tends to be complete at normal size relations of the antennal segment and body length. In this case b becomes actually an initial growth index, under the conditions established by the experiment. And so for any case, if the unit were so adjusted that the value $x=1$ coincided with the beginning of the data or some event such as hatching, or birth, or some suitable stage in the life history of the organism then the values of b could be given a definite biological meaning and could be compared directly.

An especially illuminating example is found in certain relative growth relations in macaques. Lumer and Schultz (1941) found statistically significant differences for both log b and k in *Macaca mulatta* and *M. philippinensis* during postnatal growth in the upper arm/trunk, the hand/trunk and tail/trunk relations. For easy comparison the curves for these three pairs of relations have been brought together in Figure 1. It may be seen that the curves for the two former relations are close together or tend to overlap. The curves for the tail/trunk relation on the other hand lie a considerable distance apart from each other. But the relative positions of the curves within the range of the data are not disclosed by the comparison of the values of log b for the members of each pair of curves. For the upper arm/trunk relation the values of log b are 0.2246 and 0.0074; for the hand/trunk relation, 0.1449 and 0.3239; and for the tail/trunk relation, 0.8395 and 0.6758, for *M. philippinensis* and *M. mulatta*, respectively.

The differences in log b between the two species for each relation are about the same and so give a distorted picture of the relative growth relations to be seen at a glance in Figure 1. When the measurements are converted from millimeters to decimeters, then the difference

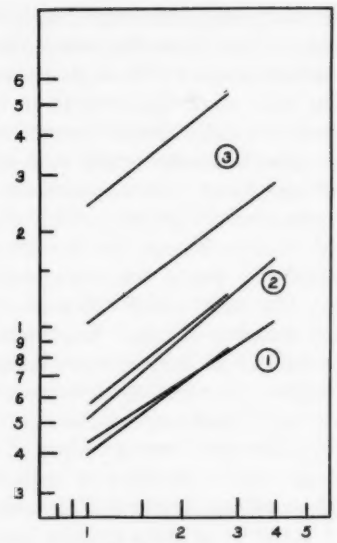

FIGURE 1

LOGARITHMIC PLOT OF THE (1) HAND/TRUNK, (2) UPPER ARM/TRUNK AND (3) TAIL/TRUNK RELATIONS IN *Macaca mulatta* AND *M. philippinensis* (DATA FROM LUMER AND SCHULTZ, 1941)

(See text for discussion.)

in log b for the upper arm/trunk relation decreases from 0.2172 to 0.053, and for the hand/trunk relation from 0.1789 to 0.0340. The difference in log b for the tail/trunk relation, however, increases from 0.1637 to 0.3615. In the two former relations the differences in log b are small and are not statistically significant because of the small size of the sample.

The main point is that now in each case b gives more nearly a true picture of the actual differences in relative growth. None of the former values, calculated in terms of millimeters, can be regarded even as a rough approximation to an "initial growth index." But for the new values of b calculated in terms of decimeters the situation is different. Although it is not strictly the initial growth index, having regard to the growth of the parts from the beginning, b is, nevertheless, the initial growth index for the postnatal cycle of growth. Since 100 mm. is approximately the trunk length at birth in these two species, b might be appropriately called the "natal growth index."

It gives directly the size of the elements y at the time of birth. The same general meaning for b can, of course, be given for any case where the curves have the same value of k but different values of b, but in these cases differences in log b are the same at all points along the curves.

It was pointed out above that when the time course of growth of x and y is exponential or parabolic then the allometric equation applies exactly. But almost always, as is well known, the time course of growth is sigmoid. This is true of morphological increase, and applies also to increase in amount of various chemical constituents, to the data of metabolism and also to certain physiological functions, for example, the gradual development of the mechanism of temperature regulation in warm blooded animals (Kendeigh and Baldwin, 1928).

The consequence of sigmoid growth for relative growth functions is rather complex (Berstein, 1934; Lumer, 1937; Pratt, 1941, see also Glaser, 1938). Lumer has investigated several of the possible cases, namely, when the time course of both x and y follow the symmetrically sigmoid or autocatalytic curve, the generalized autocatalytic and the Gompertz curves. The results show definitely that when log y is plotted against log x systematic departures from the straight line relationship occur. These departures are such as to allow an approximation to a straight line for certain more or less extended segments of the curve.

Among examples of such curves are those supplied by Paulian (1935, p. 23) for length of mandible/length of elytra in certain beetles. Here the beetles supplying the data are adult specimens of the same species which differ markedly in total size. Consequently ontogenetic growth is completed; the allometric relations are complicated in this and similar cases by the character of the metamorphosis. More pertinent examples are seen in the growth of various dimensions of the nestling house-wren (Huggins, 1940). The data show that most of the dimensions follow a full sigmoid cycle of growth from hatching until the time of leaving the nest, 15 days later. Many of the dimensions when plotted against total body length conform to the allometric equation with a marked change in slope about the 8th day. The question arises in all such cases whether the complete graph is a continuous curve or whether there is a real break. Most frequently perhaps they are simply the mathematical consequence of the sigmoid time course of x and y. But quite definite breaks perhaps are to be expected when the time course of growth shows a sequence or an

overlapping of growth cycles, as in the change from prenatal to postnatal growth, or from the embryonic to the posthatching period.

A curve which fits the consequences of sigmoid growth, but for which the time course of growth is still unknown is that showing the length of rostrum in relation to body length in the spoonbill cat, *Polyodon spathula* (Thompson, 1934). The data extend from practically the first visible appearance of the rostrum when it was only 1.5 mm.

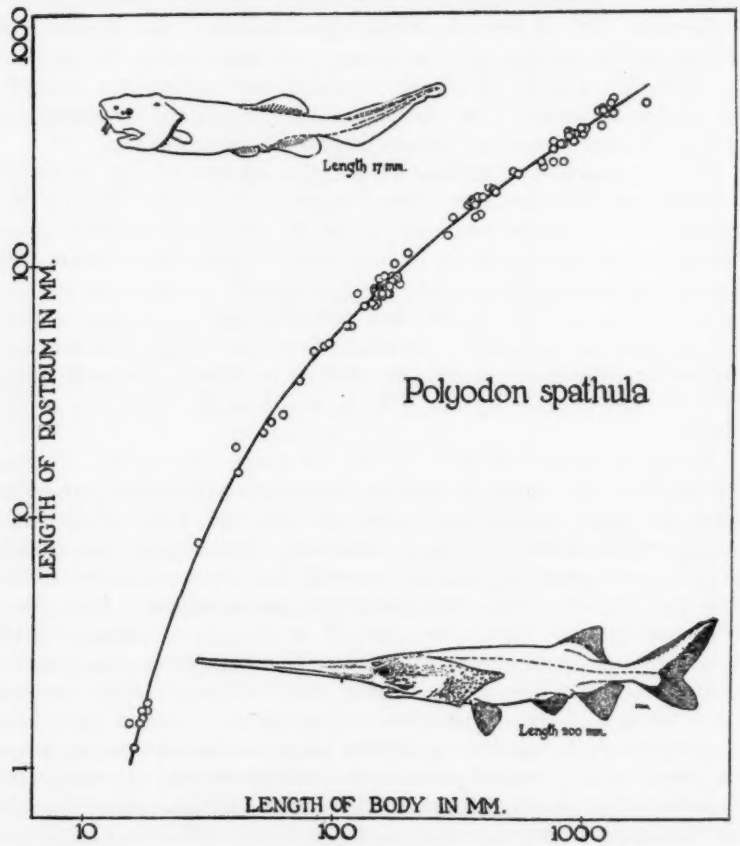

FIGURE 2
LOGARITHMIC PLOT OF ROSTRUM LENGTH/BODY LENGTH IN POLYDON
(From Thompson, 1934)

for a body length of 15.5 mm. to the largest known specimen with body length, 1727 mm. and rostrum length, 432 mm. The logarithmic plot here definitely shows a curve and a fit to several straight lines would be a forced interpretation. But it is quite clear that if the available data began at body length of about 200 mm., they could very well be interpreted as conforming to a straight line with k about 0.7. At the beginning the slope of the curve is about 4.0. If the curve should be extended downward to smaller rostral lengths, k would approach a value close to infinity. In the relatively rapid extension of the rostrum it seems certain that in the center of growth giving rise to the rostrum many successive cell divisions occur in the same plane rather than in alternate planes, and this plane is perpendicular to the long axis of the body. The same considerations apply elsewhere in the formation of any type of projection or appendage, or even in the longitudinal growth of the body itself along the main axis.

But the immediate point is that such curves as that for the rostrum length/body length relation of *Polyodon* give a clue for the widespread validity of the allometric equation as an approximation to relative growth relations in ontogenetic growth. Commonly we have but a portion of the total curve and fit a straight line through a band of points for which the true curve is a relatively flat segment of the total curve.

Since the allometric equation is an approximation, obtained in the manner indicated from statistical data, it follows that k is the slope of a regression line of log y on log x. But there are data which obviously do not conform. In such cases either growth is even more exceedingly complex than usual, or perhaps the measurements taken as x and y are not measurements of rational unitary parts given by the organism, but merely represent distances between points arbitrarily chosen for human convenience. For example, measurements taken from a point to some other point in a complicated part of the organism, such as a human or anthropoid skull, would hardly be expected to yield any significant regularity unless the measurement was in some sense a unitary element in the subpattern of the skull.

In regard to the use of equations which are empirical approximations such as the allometric equation there are two contrasting points of view. There is the attitude on the one hand which emphasizes the departures and the exceptions and, on the other, that which gives the exceptions and irregularities secondary consideration and places the emphasis on the grand sweep of the phenomenon. The history of

science shows that both points of view have their own peculiar importance in the advancement of knowledge, and the emphasis on the one to the exclusion of the other is perhaps an indication of too restricted a view. Those who emphasize the importance of the allometric equation are interested when they do so in the general trend of the main relationships which the equation brings to light in the very complicated phenomena of growth. Deviations in slope along the curve need, of course, to be taken into consideration. The slope may be at some points greater and at other points less than the slope of the entire regression line as calculated for the band of points given by the data (see Lerner, 1938).

Much more serious than the criticism that k is not constant over the entire course of growth is the one that the application of the relative growth equation to the parts of the organism involves a contradiction. For if the equation applies to the elements of a subpattern, such as the segments of a leg in relation to the whole organism, then it can not apply strictly to the subpattern as a unit in itself, i.e., to the leg as a whole, taken in relation to the entire organism (see Clark and Hersh, 1939). This may be considered at first very damning but inconsistency also has its own proper rôle to play in the advance of empirical knowledge (see Haserot's Essay, *An appraisal of consistency*, 1932).

Despite these valid objections, the relative growth function does serve, nevertheless, to give an approximate value for the order of relative growth intensities along a gradient. It is of use, furthermore, in helping to disentangle the rôle played by increase in cell number and cell enlargement, by the polarity of successive cell divisions as well as by the direction of cell extension in giving shape to the elements in the morphological pattern.

D. Relation between Ontogenetic and Evolutionary Relative Growth

In discussing above some of the points essential to a critical use of the relative growth equation there was mentioned also the relationship between the constants and change in form. The allometric equation has been applied to numerous organisms in regard to certain aspects of the total pattern but for none has it been applied over the whole course of growth from egg to adult. A relatively simple pattern which has been studied is that of the Cladoceran Daphnia (Anderson, 1932;

Anderson, Lumer, and Zupancic, 1937). The two major dimensions, total length (x) and total height (y) from the first to the 13th instar for *D. magna* and from the first to the 20th instar for the smaller species *D. pulex* conform to the relative growth equation.

Since the eggs are spherical in both species (0.25 and 0.20 mm. diameter) but at the time of the first instar length is already much greater than height, it is clear that during the embryonic period of growth the planes of cell division were more frequently at right angles to the long axis than parallel to it. From the first instar throughout nearly the entire growth period the height increases relatively faster than the length, slightly more so in *magna* than in *pulex*. The differences are not, however, completely specified by the constants of the relative growth equation. An account of the true shape requires the setting forth of the finer details. There is a greater bulge below the caudal spine in *magna* than in *pulex;* there are differences in the shape and the set of the head as well as smaller differences in general outline. The result is that while the shape changes to some extent in both species at every instar, *magna* becomes more rounded in outline than *pulex* which in contrast is more attenuated than *magna* (see Hersh and Anderson, in press).

In relative growth studies there is always the question of the relation between ontogenetic relative growth and the differences in the adult morphological pattern between related species, i.e., evolutionary relative growth. It has been found for a few types of organisms that when x and y are homologous measurements of adults from different but related species, the data likewise conform to the relative growth equation. That is, the equation is used to define allometric tribes. The main problem here is in regard to the relation between the b's and k's for the allometric tribes and the b's and k's for the ontogenetic relative growth of the representative members of such tribes. Do the values coincide or in what way do they differ? Such tribes have not as yet been studied in the Cladocera, but if the two species of Daphnia belong to the same tribe it is immediately clear they reach their final adult proportions over similar but not exactly the same ontogenetic paths. Here the b and k for the tribe differ from the ontogenetic b's and k's for both species.

More nearly typical of the degree of complexity in structural pattern among animals is that found in the hemipteran Notonecta (see Clark and Hersh, 1939). In this animal for body width, head width, and length of the three legs the relative growth equation gives a satis-

factory fit as a first approximation for the relation between length of the parts (y) and body length (x). The equation applies likewise to the subpattern of the legs in which the three distal segments were the elements studied. Since k for the various dimensions differs from unity to a greater or less extent, the parts obviously change their proportions at every instar from hatching until the adult form and size are realized. Here as elsewhere for a better understanding of the growth pattern we need to relate the posthatching cycle of relative growth to the earlier embryonic epoch.

For the posthatching phase of growth k for the legs can be used to indicate the nature of the gradient for leg growth along the thorax. the values of k for the first, second, and third legs are, respectively, 1.074, 1.069 and 0.948 for the females. Values for the males are somewhat lower. The growth equilibrium between leg length and body length indicates an antero-posterior gradient for the average along the thorax but when the individuals are examined separately it is found that only about one-half show an antero-posterior gradient, the others show a gradient highest in the middle. The dominant center for leg growth is in the tibia for the first leg, while for the second and third legs it is in the femur.

In this typical case the values of k define the nature of relative growth intensities along the gradient and indicate the dominant growth centers in the various subpatterns of the organism. Although the value of k for the third leg is 0.948 which is less than that for the first or second leg, yet the third leg might be said to be disproportionately large compared to the other legs. When final size is attained it is almost exactly twice the length of the first leg. If the 1st and 3rd legs were to continue growing at the same relative rate, the 1st leg would become as long as the 3rd leg at a body size of about 1000 mm., nearly 100 times the actual size of the full grown animal. This difference is reflected in the values of b for the leg length/body length relation. For the females these values are 0.422, 0.549, and 1.10 for the first, second, and third legs, respectively. It is clear that the values of b are an indication of the relative size of the legs at the time of hatching and so refer to leg length at the end of the embryonic period. In accordance with what was said above regarding the biological significance of b it is clear that if the unit of measurement were adjusted to the body length at the first instar, then b would give directly the size of the legs at the end of the period of embryonic growth, and so

actually be the initial growth index for the post embryonic growth period.

Notonecta is in the class of inevitable exceptions to the general rule that eggs are spherical. Its egg is about 1.6 mm. long and about 0.55 mm. wide. It seems certain that in the centers for leg growth during embryonic development there are many successive cell divisions in the same plane, and the number of such cell divisions may well be proportional to the numerical value of b. From the size relations between the egg, the legs, and the body it is clear that the relative growth curves for the legs after hatching would connect with the curves before hatching by a break or a sharp change in slope, and these curves for the embryonic period would have exceptionally high values of k. Indeed at the very beginning the curves would be expected to have values of k approaching infinity. The entire curve would very likely be similar to that for the rostrum length/body length relation in Polyodon (see Figure 2). While Notonecta gives typical results of the application of the relative growth equation to ontogenetic data, yet unfortunately, also, in Notonecta and related forms allometric tribes have not been studied. In fact, for the animals best known on the ontogenetic side there usually is little known about evolutionary relative growth, and for organisms which are known to some extent in respect to evolutionary relative growth there is usually little or nothing known about the ontogenetic growth of the representative forms.

But in any case it has been shown for many species that as the organism increases in size the relative growth equation applies extensively to data on some suitable measure of increase in many of the characteristics of the whole organism and of its parts. Similarly for a series of adults belonging to related species or genetic types, it is found that quantitative data on homologous parts conform to the same power function. (Some references which have appeared on evolutionary relative growth since the publication of Huxley's *Problems of relative growth* (1932) are: mammals, Brummelkamp, 1939; Hersh, 1932, 1934; Lumer, 1939, 1940; Lumer and Schultz, 1941; Phleger, 1940; Quiring, 1938; Robb, 1937; fowl, Lerner, 1940; snails, Hamai, 1940; Drosophila biotypes, Hersh, 1932).

In general where measurements of a series of adults belonging to related species or races have been fitted to the allometric equation it has been found that the same approximate fit to the equation occurs

as for ontogenetic data, so that the same limitations and caution in its use applies. But, of course, now the interpretations are necessarily in terms of evolutionary development, although there is also always an ontogenetic question in all such relationships.

Typical of the results is the study of Lumer (1940) on the data for the limb bones and various dimensions of the skull of some 28 modern breeds of dogs, certain prehistoric types as well as the wolf, the theoretical ancestral type. For some relations the data follow a single curve including all the breeds, for other relations two or more distinct curves. Quite consistently the results show that the various breeds can be classified into six groups, called by Lumer allometric tribes. Since allometric tribes are distinguished from one another by differences in b and in k, it seems that the conception might be of considerable use to the taxonomist, who hitherto has not attempted any considerable use of quantitative data in his scheme of classification. But, of course, it is true that species must be named before such data can be gathered. In any case the data are not sufficient as yet to test the validity of any widespread application to taxonomic problems. For the various breeds of dogs, an allometric tribe is of subspecific rank. For the data published by Osborn on the titanotheres, an allometric tribe had the rank of a genus. In this group of extinct mammals the method was able to suggest the systematic position of an aberrant specimen (see Hersh, 1934). And we might surmise that the distinguishing character or characters of any group would be found to have unusual values of k. At any rate such is the case for the frontonasal horn of the titanotheres. It has the unusually high value of 9 for the horn/basilar length relation.

On the basis of values of b and k, several possibilities are to be recognized, namely, convergent, divergent, and parallel allometry; and a fourth irregular type, composed of some combination of the other three types. In the case of the dogs, while there are some irregularities, there is in general convergent allometry of the tribes, with the wolf representing the point of convergence and obviously confirming in allometric terms the view that in the evolutionary development of the various breeds of dogs, selection in general was for mutations for smaller skull dimensions than those characteristic of the wolf. For example, within the terrior tribe the proportions of the parts of the skull become more and more similar to those of the wolf as we pass from smaller to larger breeds within the tribe and so for other

tribes which converge toward the wolf in respect to various size relations of the skull.

For the titanotheres, so far as the main dimensions of the skull are concerned, there was in general a divergent allometry in Eocene times with a tendency toward parallel allometry for the dominant genera Palaeosyops and Dolichorhinus. This was followed in Oligocene times with a tendency toward an irregular or convergent allometry as the group approached extinction.

A remarkable example of persistent parallel allometry has been found by Brummelkamp (1939) in the brain weight/body weight relation in various groups of vertebrates including fish, amphibia, and mammals. In each group the points for the various species fall along or very close to a series of parallel lines conforming to the allometric equation, with k for all equal to 5/9 and for which the b's differ from one another by some integral power of the $\sqrt{2}$.

As mentioned above a main problem in regard to allometric tribes is to find whether the ontogenetic b's and k's for the various members of the tribe do or do not coincide with the b and k of the tribe. It happens that there are some data, rather inadequate for leading to conclusive results, on the German Shepherd dog which allow an esti-

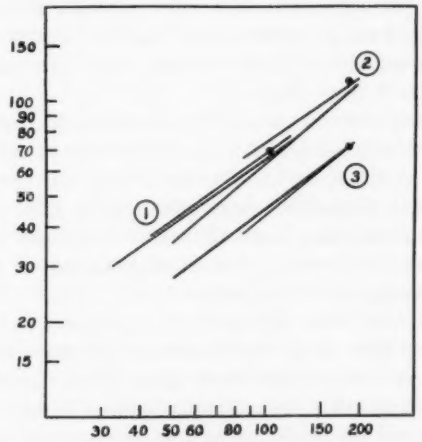

FIGURE 3

LOGARITHMIC PLOT OF (1) PALATE WIDTH/PALATE LENGTH, (2) ZYGOMATIC WIDTH/BASILAR LENGTH, (3) OCCIPITAL WIDTH/BASILAR LENGTH, FOR THE TERRIER TRIBE AND FOR THE ONTOGENETIC RELATIONS IN THE GERMAN SHEPHERD DOG
The shorter line of each pair is for the terrier tribe on which the position of the German Shepherd dog is marked by a closed circle.

mate of the ontogenetic b and k for certain dimensions of the skull. Dr. Lumer has kindly supplied the necessary data for Figure 3 which shows the degree of correspondence for the German Shepherd dog and the terrier tribe to which it belongs. It can be seen that the curves do not coincide exactly. For all the several relations studied it may be said that the points for the tribe and the points for the ontogenetic data on the German Shepherd dog fall within the same narrow band, and so sensibly coincide, although the actual numerical values of the constants, calculated by least squares from the meager, and perhaps not entirely comparable, data do not agree within the limits of statistical significance, except for the palate width/palate length and the snout length/lower jaw relation (see Lumer, 1940, Table 3).

In the present state of knowledge there is clearly not sufficient grounds either way for saying whether or not the ontogenetic growth relations of members within the same tribe generally coincide. But where there is such coincidence then the biological significance is simply that within the tribe members differ from one another in genes for general size irrespective of what changes in proportion might be shown from a comparison of the various indices in respect to intratribal variation in adult proportions. That is, differences between representatives of the same tribe are merely differences in absolute size and changes in proportions between adults of the different representative members will occur when k for the various allometric relationships differs from unity.

It is plausible to assume that such size differences between members of the same tribe ultimately reduce to differences in cell numbers along the dimensions that conform to the relative growth equation. That is, the genes in which they differ bring about their effects by altering the number of cell divisions. These genes for increase in general body size do not alter the general plan of distribution of the cells to the elements in the organismic pattern.

Furthermore, one tribe differs from another by constant genetic differences which alter in a more fundamental way the morphological pattern. Here too the problem is to disentangle the parts played by differences in cell number and cell enlargement as well as the direction of cell division and the direction of cell enlargement (see Sinnott and Dunn, 1935).

But there is an additional problem in all such cases. Representatives of different allometric tribes, but of the same body size, have undergone the same amount of growth, so that if one organ or element in

the pattern is larger than the homologous element of the representative of the second tribe, it would necessarily follow that the distribution of growth to some of the other homologous organs would also differ.

This raises the question of the relationship between the homologous elements and the relative amounts of tissue participating in the embryonic induction which gave rise to the homologous parts, as well as the relation of the difference in size to the increase in cell number after the embryonic determination of the parts. It may possibly be that differences in b and in k between allometric tribes represent a difference in the timing of the embryonic inductions for the homologous organs.

Many of the points discussed may be graphically summarized. Figure 4 (for which I am indebted to Dr. B. G. Anderson) is intended as a hypothetical diagram of a few of the possibilities allowed by the present state of knowledge. In Figure 4 the field of data is indicated by points through which two sets of parallel lines are drawn, one set of lesser slope, with k about 0.67 and the other set with slope about 1.50. Taking the data first as representing ontogenetic relations, the three top lines typify the relations found in regard to the three main dimensions of the head relative to increase in body length for the nestling house wren. This low value compensates for the greater slope before hatching as shown by the lines continued toward the left. Approximately the same type of relations hold if now the three top lines are taken to represent the three legs of Notonecta, although in this case the lines are not parallel. The height/length relations in different species of Daphnia before the first instar are represented by the lines which start with smaller slope but which after the first instar have a slope greater than one. The lines may also represent the data on ventral and dorsal facet number in a series of bar-eyed Drosophila stocks raised at various temperatures, which are discussed below.

If now the diagram is taken to represent a series of allometric tribes, only the case of parallel allometry is indicated. As discussed above the question in all such cases is whether the b and k for the tribe coincide with the ontogenetic b and k for the various representative members of each tribe. If so, then the lines, let us say of smaller slope, are at one and the same time lines for the ontogenetic relations as well as for the allometric tribes, but if not, then the ontogenetic relations are indicated by lines of different slope than those indicating the tribes.

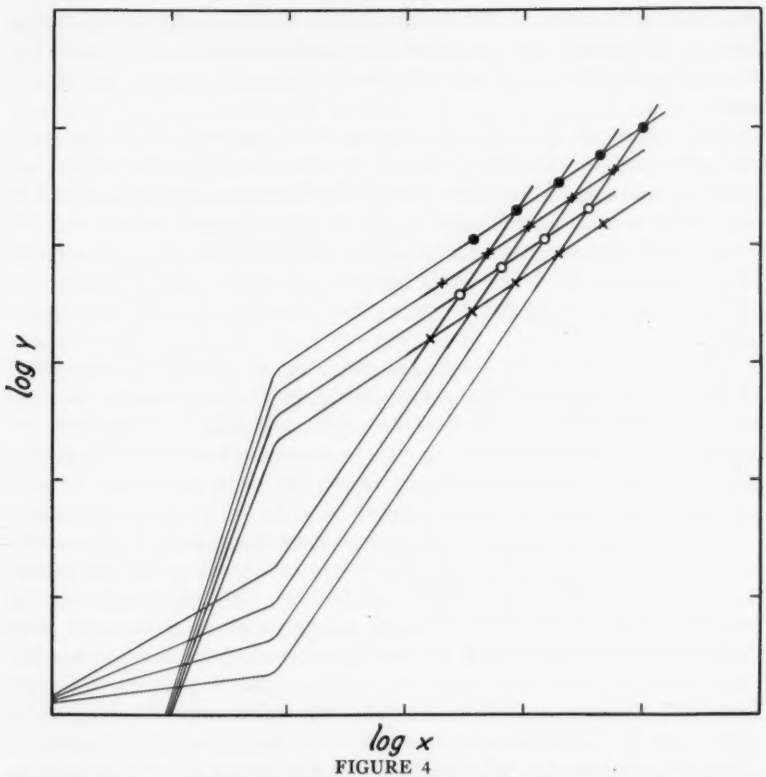

FIGURE 4

HYPOTHETICAL LOGARITHMIC PLOT INTENDED TO ILLUSTRATE ONTOGENETIC AND EVOLUTIONARY RELATIVE GROWTH AND THE WAY IN WHICH THEY MAY BE RELATED TO EACH OTHER

The field of data is indicated by circles and crosses, through which two sets of parallel lines have been drawn, with values of k about 0.67 and 1.50. See text for further explanation.

This attempt to explain the relationship between ontogenetic and evolutionary relative growth shows clearly that the necessary data to fully illuminate the problem are still lacking, but it nevertheless indicates the problem involved. Much that falls under the terms, "differential growth" and "morphological pattern" is very inadequately treated by the relative growth equation. That equation after all gives us merely an approximate description of the quantitative size relations as the organism increases in size during ontogeny, or of the quantita-

tive relations between homologous elements in an evolutionary series. It does not tell us anything about the origin of the elements in the morphological pattern or of the physiological mechanism by which the particular size relations are reached and maintained in the organism.

A good example to show that very diverse final subpatterns may result from the same sort of anlagen is found in the external genitalia. Here it is well known that the anlagen of male and female genitalia are much the same but the difference in the subsequent relative growth of the parts result at maturity in size relations between the elements in the subpatterns which completely mask the fundamental similarity of the original pattern. In this example the elements of the subpattern are present at the completion of growth which were present at the beginning but there is great disparity in the relative size of homologous parts. There are other cases where elements are suppressed or lost from the original pattern as laid down at the beginning, as in the staminate and pistillate spikelets in maize (see Weatherwax, 1923, p. 120). Here likewise the primordia at the beginning are the same but homologous elements in the two cases have diverse growth histories which result in final subpatterns at maturity so diverse as the tassel and the ear of the maize plant. The parts which actually arise and then are suppressed may be regarded as centers of defective growth.

These are cases of normal growth relations. An abnormal pattern of an extreme sort, but of the same general type, at least in the sense that parts of the normal pattern fail to come to full growth is that of the otocephalic guineapigs (Wright and Wagner, 1934) which are approximately normal in body size but markedly defective at the anterior end. The question naturally arises whether in these cases we are concerned with a failure of embryonic induction or whether the anlagen are normally induced but the centers for the parts are defective in their growth capacity.

The problem of the relative size and shape of parts in the development of the individual and the race can no doubt be clarified by such examples where size differences are considerable. In the development of the individual such cases are usually called arrests in development or localized dwarfism, and in discussions of evolution are usually referred to as rudimentation (see Goldschmidt, 1938, 1940). Localized dwarfism is apparently only an extreme instance of a more general phenomenon, and if so, the results could be generalized to include the

more common phenomenon of less extreme differences in size and shape of homologous organs between genetically related forms.

Without attempting to make a detailed classification, there are at least two main types of localized dwarfism; one which is brought about by an actual loss of parts and a second type in which the apparent loss is simply an agenesis brought about by deficient growth or defective embryonic induction. If a part arising early is necessary for a later induction, then the later induction may fail to occur when the part arising earlier is a center of deficient growth. Briefly, in this second type of localized dwarfism, the question is, do the parts arise and fail to grow, or do they fail to appear as the result of a primary failure in the induction process?

At any rate it is clear that the problem of relative size differences, whether such differences are large or small, would be better understood if the ontogenetic problem were solved for some one single element in a pattern whose growth relations were known from the standpoint of genetic and environmental control and which was known also from the standpoint of descriptive and experimental embryology. There is, of course, no such completely worked out case. But it might be of interest to attempt to sketch the present state of knowledge about some one element, such as the eye of *Drosophila melanogaster*. Here the size of the part can be modified considerably by genetic and environmental factors, with no great change in the rest of the pattern. The size of the eye grades from the full size of the wild type down through a series of decreasing facet number to the localized dwarfism found in ultra bar.

In the bar series the data on average facet number of flies with different genetic constitution at the bar locus conform to the relative growth equation for the dorsal and ventral lobes of the faceted area (Hersh, 1928). When the developmental temperature changes, the size relations between the dorsal and ventral lobes also change, but again in conformity with the relative growth equation. When the genetic constitution is held constant the value of k is about 1.50 and b increases with every addition of bar factors, that is, as the order of eye size decreases. On the other hand, when the temperature is held constant and the genetic constitution at the bar locus made to vary, then the value of k is about 0.8, although the fit to the equation is not very good at the higher temperatures; b now increases as the temperature decreases. That is, as the order of eye size increases with the decrease in temperature the value of b increases. The results show that the

dorsal and ventral lobes of the eye are modified in a perfectly regular manner by change in genetic constitution and by change in temperature at which the flies are raised, and these two series, the genetic series and the temperature series, conform to the relative growth equation (see Figure 4). Furthermore, when a homozygous bar stock was systematically modified by inserting the recessive genes of the Xple stock singly and in various combinations for a total of 15 biotypes, it was found (Hersh, 1931) that the dorsal and ventral lobes within any biotype likewise conform to the relative growth equation when the data are seriated on the basis of total eye size. The values of k range from about 0.66 to about 1.50. Now it is to be remembered that k is a ratio which tells nothing directly of the absolute rates, i.e., k would have the same value if the absolute rates were as 1:2 or as 10:20. If the absolute rates were as 6:8 the value of k would be higher than when the absolute rates were as 10:20. And it is perhaps important in this case as an indication of changing absolute rates, that as k increases the average facet number for the 15 stocks swings alternately above and below a line in quite regular manner so that the result is a periodic curve when facet number is plotted against k (see Hersh, 1931, Figure 9). Also for the same data the 15 biotypes fall into three allometric tribes, in which there is no obvious relation to the ontogenetic values of k for the biotypes composing the separate tribes or in regard to the distribution of the Xple genes.

We have here conformity of the data to the relative growth equation with ontogenetic implications and for a series of genetically related biotypes in the manner of evolutionary allometry. In regard to the more strictly embryological aspects it is known that in the egg of Drosophila there is a cytoplasmic area whose prospective potency includes an organ-forming region for the eye. But this region does not control the size of the eye of the adult, although it may possibly be concerned with fixing the upper limit of the number of facets in the compound eye and may have smaller secondary effects on the size of the eye in the bar series. Another embryological phase of eye development is known through the work on temperature effective periods. While at present the status of this period in relation to the growth of the optic disc and to differences in final eye size is somewhat uncertain, nevertheless the temperature effective period is one link in the development of the eye.

At any rate the *TEP* in the case of wild type extends throughout the whole period of larval development (Margolis and Robertson,

1937). The *TEP* for the other members of the series for which it has been determined occupies a more restricted period during the later larval life. It seems to occupy a part of the third instar, at least at the higher temperatures, for bar, ultrabar, and infrabar (see Driver, 1931; Luce, 1931; see also, Hersh, 1934). From the manner of its determination the *TEP* isolates a period in the development of the eye which has a different temperature coefficient than the other processes occurring during development.

The final eye size is not only affected by altering the genetic constitution and by temperature transfers at a critical time during development. The eye size can be altered by a change in the genetic constitution during the course of development, as shown by a study of eye mosaics in flies of suitable constitution. This change at the bar locus during development may be for a larger or for a smaller eye, depending upon whether the genes for eye color are or are not on the same chromosome with the bar factors.

The data on such mosaics show that the number of white facets (y) in the mosaic eye when plotted against the total number of facets (x) conform to the relative growth equation. When the change in genetic constitution during development is for a larger eye then the value of k is nearly 4, indicating that as the eye increases in size the number of white facets increases about four times over that of the total eye, so that the eye will be expected to be all white at an eye size smaller than the wild type (see Hersh and DeMarinis, 1938).

The size of bar eye can also be increased by the feeding of an extract of Calliphora larvae (Ephrussi, Khouvine and Chevais, 1938) which therefore contains the B^+-substance or the hormone whose elaboration is definitely related in some manner to the bar locus. In this case the number of facets increases directly with the concentration of the substance and is effective during a period which corresponds quite well with the time of the temperature effective period (Chevais, 1939).

All these various data may be brought under a common point of view if we make the simple assumption that the factors at the bar locus are concerned in some way with the elaboration of a substance which is an intra-cellular activator of cell division in the optic disk. If the B^+-substance is present beyond a certain necessary concentration for cell division to occur, it may diffuse into surrounding tissue to activate cells which are deficient in it because of the presence in them of some one or more of the bar factors. These various data

are more consistent with the view that the localized dwarfism of the bar series is an agenesis rather than an actual loss from the pattern as Goldschmidt and Höner (1937, Figure 2) have found for the case of vestigial wing.

But the main difficulty regarding the problem of differential growth remains; how does it happen that the action is primarily localized in the optic disk? This is an aspect of the time honored problem of how the organism controls the origin and the size of its parts. But since in problems of differential growth we are concerned with increase in size and in relative size of the parts, the problem of localization involved reduces itself to the pattern of cell division and cell enlargement.

The centers of growth, which give rise to the parts in the anatomical pattern, are of course differentiating centers with regard to the organism. These centers of growth may give rise to solid or hollow extensions or to solid involutions or hollow invaginations, depending apparently on the position of the spindle in successive cell divisions. The problem of localization is of course one of the oldest in modern embryology, and in making the swing around the circle we are in contact with it on a new and more promising level. On the basis of quantitative experimental data, which conform to the allometric equation, it can now be investigated in terms of the concepts of genetics, embryology, and biochemistry. One gets the impression that data will perhaps soon be available for a clearer understanding, at least in outline (see Waddington, 1940), of the relation between genes, organizers, and hormones, and the way in which they are related to the origin, regulation, and maintenance of the morphological pattern.

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ABNORMAL GROWTH PATTERNS: WITH SPECIAL REFERENCE TO GENETICALLY DETERMINED DEVIATIONS IN EARLY DEVELOPMENT

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The pattern of growth and development may be viewed, for the purposes of this discussion, as the order which emerges early in the life of the individual, and within which the main parts and relations of the body are foreshadowed. A general pattern of this sort is characteristic of each group of organisms. It represents the response of a specific hereditary constitution to the stimuli and substances of the external world. A deviation from normal of either of these components relative to the other, that is, a sudden new change in the hereditary constitution or a radical change in the environment, may therefore be expected to lead to changes in patterns which express the adjustment of the organism to its past environments. When the changed response leads to some wide departure from the form characteristic of the species we call the result abnormal and consider the process which led to it an abnormal process.

The analytical study of such deviations has played a large rôle in our understanding of development. The major interest has been in the response of the developing organism to some changed condition of growth, for by noting what is changed in passing from normal to abnormal development, we learn to identify the factors which control development. The modification of the response has usually been effected by changing specified parts of the environment, or by changing the relationships of the parts of the developing organism to each other, and most of our experimental knowledge of the factors controlling growth patterns comes from these sources.

It is possible now to supplement the information and test the conclusions obtained by these means by a study of those cases in which the internal component, the hereditary constitution, has been changed by mutation. New interest attaches to such studies because we have come to recognize the importance of a fact which has often been overlooked, namely, that new alterations in genes or chromosomes lead to just the same types of abnormalities as changes in specific elements of the environment. It seems quite clear that if each developmental

response is conditioned by mutual relations between a specific heredity and a specific environment, then failure or deficiency of either one must lead to the same result. So similar are the abnormalities resulting from these two types of change that it is generally impossible to tell, in advance of an experimental study, which was primarily responsible. There are many examples of this of which only one need be cited. Congenital absence of the tail occurs in nearly all tailed vertebrates sometimes as a sporadic abnormality, and sometimes as a mutant form which may come to characterize whole breeds and varieties as in the tailless cats, dogs, sheep, mice, and other mammals. Both forms are found in the fowl, where among the normally tailed breeds occasional rumpless individuals are found which do not transmit the defect, while other fowls with the same character transmit a mutant gene in the usual way. In the sporadic form the abnormality is probably due to a disturbance in the early embryo. Danforth has obtained rumpless embryos from eggs subjected to abnormal temperatures in the early days of incubation, while Dr. Landauer informs me that the frequency of rumpless embryos is increased among eggs shaken before incubation. One would guess that there are various ways in which the growth of the tail region might become abnormal and that whether the disturbance originates within or without the embryo, the pattern would suffer a similar distortion. The abnormality arising from the gene alteration has been called hereditary, while that which occurs sporadically or following external modification is classed as non-hereditary. We can no longer regard these as sharply alternate categories. Some new results which we have just obtained show that both are hereditary.

We have studied the frequency of taillessness in three closely inbred and generally homozygous families of rats. In one of these congenital taillessness appears in about one per cent of the offspring; in another line, in about two per cent; and in a third line in about four per cent. In the general population of laboratory rats only 19 sporadic cases of congenital taillessness have been listed in the literature, out of over 170,000 births observed (King). These lines differ from the general population and from each other in the tendency to produce tailless young. This tendency is therefore due to the genetic constitution of the individual inbred line. But since all the individuals of each line presumably have the same genetic constitution, we should not expect tailless and normals within the same line to show any genetic differ-

ence. It has been possible to test this assumption in the line which produces four per cent of tailless young. Here we find that matings of tailless by tailless, tailless by normal, and normal by normal from tailless parents or from normal parents, all produce the same result, that is normal offspring, subject of course to the four per cent chance of taillessness which obtains within this line. Here we have the case which bridges the gap between the hereditary and the apparently non-hereditary. The genetic constitution of the race determines the likelihood that an embryonic growth process will go wrong; but each occurrence of such an error is, so to speak, a separate accident. In this case, as in the occurrence of otocephaly in guinea pigs described by Wright, the genetic constitution sets the threshhold of a response to some developmental accident which is the proximate cause of the abnormality. In those cases in which *all* offspring inherit a mutant gene and *all* become tailless, one may wonder whether the gene does more than change the threshold to such a point that the vagaries of the usual environment will always evoke the response.

It is precisely because heredity and environment are thus interdependent, that nearly all new genetic changes have one effect in common, to reduce the efficiency of growth processes and to render the operations of development less stable and more susceptible of being changed by the minor vagaries of the environment. Thus nearly all new mutations should provide material for the study of abnormal growth processes.

Most mutations hitherto described and dealt with by geneticists represent minor modifications in developmental processes. They have, of course, been chosen for study because they do not seriously impair the viability. But these represent only what Muller has called the narrow visible part of the whole spectrum of mutative changes. The largest number of mutations either have effects so minor that they are not noted or so severe as to stop development. The latter are the lethals and it is among them that we may expect to find the best material for the study of abnormal growth patterns.

In the types of changes in development which may occur we might be tempted to recognize problems of two different magnitudes. One concerns the way in which a single mutation, for example that responsible for taillessness produces a difference in development, and this is generally addressed to the geneticist. Another and larger problem concerns the ontogenetic origin of the patterns by which large groups of organisms differ, for example the tailed and tailless am-

phibia. This is the type of developmental difference with which embryologists are called upon to deal, and it has been supposed to differ from the simpler problem of the geneticist not only in magnitude but in kind; since it has not appeared to be subject to mutation, it has seemed to belong in a different category altogether.

There is no doubt that this is a more difficult question than the one presented to the geneticist, but it seems to me no longer reasonable to regard the two problems as differing in any fundamental way. We know, for example, mutations which alter such "fundamental" characters as the metameric structure of a mammalian embryo; of others which suppress entirely the formation of a whole germ layer; while still others alter the first movements of cleavage nuclei upon which the primary organization of the embryo depends.

In development, the distinction between fundamental and derived characters, between patterns of a phyletic, generic, specific, or "mere Mendelian" level seem now as artificial as the sharp categories which for so long stood in the way of any advances in our understanding of evolution. It is quite true that mutational changes which upset early pattern processes all act as early lethals, but they are connected by a continuous intergradation with those mutations which produce only minor alterations in the details of developed structures. That the early changes are lethal is not a reason for dismissing them as mere pathological freaks, but much more an invitation to determine in what ways the normal pattern is upset, and what its interdependencies are which are separately changeable, and out of the relationships found to construct in these terms some more general view of development. This is not very different from the method of Entwicklungsmechanik. Whereas the experimental morphologist changes the organism deliberately and from the outside, the geneticist must await an act of God (or of short wave radiation) to change the organism from the inside. He may however be rewarded by obtaining changes, especially in mammals, which it has not yet been possible to produce by operative methods. It is not surprising, therefore, that increasing attention has been paid to lethal mutations and that attempts have been made to trace the changes they produce to their developmental origins.

Many such studies have been carried out during the past few years, but in relatively few have the early disturbances in growth patterns been described and in still fewer have the theories relating the changed pattern to the primary genetic change on the one hand, and to the final form on the other, been subjected to experimental test. Landauer in

a recent paper has discussed those cases in which hereditary abnormalities may be assumed to take their origin from effects of factors of a general nature which act on the embryo as a whole. The genetical archetype illustrating this interpretation is disproportionate dwarfism of the fowl, especially that found in connection with the creeper mutation which has been so thoroughly investigated by Landauer and his collaborators. What one might call the growth or gradient theory as applied in this case assumes that the differences in the localized effects of a mutation (the greater shortening of legs than of wings in chondrodystrophy, for example) are due to differences in the response of the different anlagen to an over-all change in the intensity of growth. This idea was first applied to a general category of results by Stockard who showed that external agents of many kinds which had an inhibiting effect on growth would, when applied at a critical stage of development, produce the greatest reduction in just those parts which were at this time undergoing the most rapid growth. Among the ancestors of this theory the gradient ideas developed by Child are readily recognizable, and in modified form it has been applied by Wright in explaining the origin of the otocephalic series of malformations and by Scott in the case of lethal polydactyly in guinea pigs.

Landauer has pointed out the existence of teratological correlations found in large groups of animals, such as the frequent association of abnormalities of the head and extremities among lethal mutations in the fowl, and the abnormal syndromes of associated defects found in man and other mammals. The parts similarly affected are those which had simultaneous peaks of growth activity at a time when the growth of the whole embryo was retarded.

There is no doubt that this interpretation gives a reasonable view of some of the developmental steps by which many structural abnormalities arise, both those due to mutation and to environmental modification. But, as Landauer points out, such "general" factors as reduction in growth may themselves have more "specific" causes which are still to be sought in earlier stages. This is merely to say that a growth or gradient theory would not solve the whole problem. In the second place it is not clear that any fundamental changes in the developmental pattern of the whole embryo are produced by genetic changes with effects like those of the creeper mutation. The theory in fact assumes the persistence of a general basic pattern which determines the differing responses of the parts to general agencies.

In a revision of earlier views Landauer (1941) has recently suggested that the parts differ quantitatively in their physiological needs as imposed by differential growth activity and by similar differences. On this view alteration by mutation of a developmental process common to the whole embryo, should lead, as in the case of disproportionate dwarfism to abnormalities both in size relations and in structural characters.

In addition to mutations of the sort discussed above, there are a number of mutant effects in which general modifications in the developing embryo appear only after more localized and specific changes have become evident. Some of these have been shown to effect radical changes in the early patterns of growth. An exhaustive consideration of these would require citations of examples from all the classes of vertebrates and from many invertebrate groups; and an evaluation of the evidence of genetically abnormal growth patterns in plants. The time hardly seems ripe for such a discussion. Instead, I shall arbitrarily limit myself to a discussion and comparison of three sets of observations in which embryological and experimental methods have been applied to the early stages of genetically abnormal forms. One of these deals with an insect, *Drosophila melanogaster;* one with a number of related mutations in a mammal, the common house mouse; and the last concerns the peculiar lethality associated with species and generic hybridization in newts and salamanders.

By the use of lethal chromosome deficiencies Poulson has been able to demonstrate some of the processes through which the insect egg normally exerts control over its growth pattern. In *Drosophila melanogaster* it is possible by making appropriate crosses between mutant stocks to obtain (*a*) fertilized eggs which entirely lack the X-chromosome (referred to as Nullo-X); (*b*) fertilized eggs which lack the right half or (*c*) the left half of the X; (*d*) which lack a small section of the X-chromosome, Notch deficiency. The classes of zygotes so produced differ from each other and from the normal in the quantity of X-chromosome material, hence the number of X-chromosome genes which they contain.

Where the whole X-chromosome is absent the first nuclear divisions occur but the resulting nuclei, instead of migrating to the periphery of the egg to form the blastoderm, tend to remain clumped in the anterior half of the egg and to form cells there. In the anterior cap the nuclei undergo abnormal mitoses in which cytoplasmic division fails to keep pace with nuclear division resulting in clumps of

polyploid cells. The final result of these and other abnormal processes is the production of a stratified mosaic zygote with cell mass at anterior end, cytoplasm in center and yolk at posterior end. The abnormal processes begin at the end of the first hour of development, and pycnosis and death set in at about 10 hours, so that this deficiency acts as a very early lethal. It is noteworthy however that the eggs which are to die have their own characteristic pattern by which they can be readily recognized. The chief failure appears to be in the growth movements of the cleavage nuclei, while other effects may be secondary to this.

When half of the X-chromosome is missing development proceeds somewhat farther. The cleavage nuclei are formed and migrate more or less normally. When it is the right half of the *X* which is missing, blastoderm formation is not complete; no nuclei invade the posterior pole and consequently no cells are formed there. When it is the left half of the *X* which is missing, a more or less regular blastoderm is formed, but cellular irregularities arise within the blastoderm and at gastrulation the germ layers fail to separate, leaving an undifferentiated mass of cells surrounding the yolk. Cell division continues, but the cells apparently are unable to use the yolk material, dwindle in size and degenerate. In both cases the chief departure is failure to form the normal germ layers and subsequent growth therefore fails to contribute to differentiation of organs.

Poulson has also studied several different deficiencies for the Notch region near one end of the X-chromosome. Males receiving such a deficient X-chromosome lack from a few to many of the genes normally present in this region. Such male zygotes all show the same developmental defects regardless of the length of chromosome which is missing. They die as embryos but at a later stage than those which lack a whole or a half chromosome. The early steps in development are carried out normally and a normal germ band is formed, but when the time arrives for the nervous tissue to separate from the hypoderm, this fails to occur; the nervous tissues grow at the expense of all other cells forming a large neural mass by which such embryos may be recognized. The cells which should become mesoderm instead become nervous tissue, while the anterior ectodermal derivatives (salivaries, imaginal disks, etc.) fail to appear. Here gastrulation is carried out, but one of its important products—mesoderm—does not appear.

One smaller deficiency (Scute-8) has also been studied by Poulson

and is found to die at the end of the embryonic stages with minor defects in muscles and tracheae. Finally Kaliss in our laboratory has studied a still smaller deficiency which lacks only a few genes. This dies as a fully formed embryo which however fails to hatch from the egg. Morphologically it is normal but there are indications that the interchanges of gas and fluid through its membranes are abnormal.

Here are convincing proofs that the processes of early development in the insect egg are under the control of its own genes. There is a rough correspondence between the number of genes missing and the stage at which normal control is lost (Table 1). This, as Poulson

TABLE 1*

SUMMARY OF EFFECTS OF THE SERIES OF X-CHROMOSOME DEFICIENCIES DESCRIBED IN THE TEXT

Deficiency	Size	Time of action	Effects
Nullo-X	Entire X	1st hour	Abnormal distribution of nuclei; no blastoderm
Half-X	Half of X		
1st type	XR	1st-2nd hours	Incomplete blastoderm
2nd type	XL	2nd-3rd hours	Fails to separate germ layers; no differentiation
Notch-8	Includes bands 3 C 1 to 3 D 6	6th hour on	Nervous system fails to separate from rest of ectoderm and hypertrophies. Mesodermal organs lacking. Fore-gut rudimentary; mid-gut incomplete
Scute-8	y H w ac missing at tip of X	Late embryonic stages	Nearly complete larva; no air in tracheae; little or no muscular movement

*Poulson, D. F., 1940. Table 4, page 313.

points out, is to be expected if many genes concerned with pattern formation and growth are distributed at random throughout the chromosomes. Loss of a whole or a half chromosome would certainly entail loss of many important genes, while a certain small section might include one or more, whereas a somewhat longer section elsewhere might by chance contain none of primary importance for early processes. The quantity relation between completeness of chromosome complement and degree of control of developmental processes does not therefore extend to small segments.

These observations suggest, too, that the developing insect egg is a mosaic in a sense other than that in which this term has been used. The growth pattern characteristic of the species does actually consist of parts in the control of which a degree of separateness is possible. Cell division and cell formation may proceed to some extent

independently of the molding of these elements into germ layers and organs, although eventually a bad pattern appears to deprive the cells themselves of access to food materials and to doom the whole organism. The observations on late embryo lethals and on homozygous deficiencies which die in larval stages, such as the Minutes studied by Brehme, show that the genes which are missing in these cases are not necessary for the control of earlier processes, and that later growth is to this extent independent of earlier events.

Evidence of a similar sort relating abnormal growth patterns in vertebrates to specific genetic conditions has been obtained during the past eight years. In the Genetics Laboratory at Columbia University we have been studying the effect on early development of mutant changes at one locus[1] in the house mouse. We have studied five different mutations, all of which behave in inheritance as alternative changes at this locus, all of which act as lethals or reduce viability in the homozygote and all of which disturb the pattern of early growth and differentiation in similar ways. Changes in these earlier processes result in modifications in later structures and functions which depend upon the first; and a study of the relationship between the secondary changes should point to the channels through which genic control of development is effected. Although our evidence is not yet complete enough to permit any final answers to such questions, we have been able to narrow the problem somewhat and to ask: What is the relation between the manifold effects on development which follow from several changes at the same locus? Are they as often assumed, merely quantitative changes in one general pattern process which proceeds to a greater or lesser degree as the allelic forms of that locus are more or less different? Or are fundamentally different processes affected by alleles of the same gene?

The main morphological and viability effects of combinations of these genes are outlined in Table 2. In addition there are other effects of great importance and interest which must be omitted in the present context. Of the five mutations represented, three are dominant, and two are recessive. (A third recessive, t^2, probably represents a recurrence of the t^1 mutation and has not been studied in detail). All of them share certain peculiarities. All except one (T^f) are lethal when homozygous, and concerning this one homozygote very little is known as yet for it is very rarely found and probably suffers a high mortality just after birth. The stage at which the embryo dies is as characteristic for each mutation as is the visible

[1]See footnote to Table 2.

TABLE 2

EFFECTS OF MUTATIONS AT *T* LOCUS IN THE HOUSE MOUSE†

Homozygotes				Compounds			
Genotype	Viability	Tail	Other effects	Genotype	Viability	Tail	Other effects
T^fT^f	poor	fused	anaemia?	T^fT	viable	fused	
				T^fT^k	?	?	waltzing
				T^f+	viable	fused	deafness
T^kT^k	embryonic lethal	kinked		T^ft^o	viable	fused	spina bifida
				T^ft^1	viable	fused	
			notochord				
TT	lethal 11 days	absent	somites and posterior parts missing	T^kT	*viable	kink	chorea
				T^k+	viable	kink	deafness
				T^kt^o	*viable	kink	spina bifida
				T^kt^1	*viable	kink	
t^ot^o	lethal 5 days	—	no mesoderm				
				$T+$	viable	short	posterior tail lacks notochord and degenerates
				Tt^o	*viable	tailless	
t^1t^1	lethal<5 days	—	fails to implant	Tt^1	*viable	tailless	
				t^1t^o	*viable	normal	♂sterile

*Viable compounds of two lethals.

†Since this paper was presented evidence has been obtained by Caspari and Dunn (unpublished) that a crossing over sometimes occurs between T^k and T and thus that these mutations, although apparently near together, are not allelic. Similarly Dunn has observed crossing over between T^f and T. Tt^o and t^1 have shown no crossing over.

morphological effect, and the lethal periods within this one series cover practically the whole range of development from pre-implantation to post-natal stages. This fact alone should make one chary of making sharp distinctions between fundamental and superficial character effects since all sorts may be due to mutations at the same locus. All the recessives alter the segregation ratios in the male, probably through an effect on spermatogenesis; but none of the dominants do this. All in some combination affect the tail and axial skeleton possibly through altering the same or similar growth processes in the embryo. A peculiar behavior like that of the Japanese waltzing mouse is associated with two of the dominants although this is irregular in its expression; when present it is generally accompanied by deafness. Incomplete closure of the spinal cord, producing spina bifida, is of frequent occurrence in connection with T^{t} and T^{tk} and is probably regularly present to a slight degree in the tailless animals arising from combinations of *T*.

The similarities in morphological effect are probably consequences of interference with the same chain of normal induction relationships in the early embryo. We owe the first proof of a relationship of this kind to Chesley who in 1932 showed that the homozygote of the *T* mutation lacks notochord, somites, and all posterior structures (hind limbs, tail, etc.). The discovery of pycnotic nuclei in early stages suggested that the posterior derivatives of the primitive streak were abnormal and that all structures dependent on these lacked the induction stimulus for further growth, and either failed to develop or developed abnormally. This was borne out by the condition found in the heterozygotes, since wherever the notochord was missing the tail failed to continue growth and was resorbed, producing the short-tailed phenotype. Similar facts were established for the tailless forms by Gluecksohn, who also showed that in the t^{o} homozygote which dies shortly after implantation, no mesoderm is formed. In the mouse, mesoderm and notochord take origin together during gastrulation, and it is not difficult to see in the failure of the mesoderm an earlier manifestation of the abnormality which prevents formation of the normal notochord. The t^{1} homozygote dies at so early a stage that it is not possible to specify in what way its growth has been stopped. In combination all of these mutations reinforce each other or at least do not minimize each other's effects (t^{0} for example modifies *T* from short tail to tailless).

These effects resulting in morphological modifications can be inter-

preted as being due to quantitative changes in processes of the organizer type, all of the mutations at this locus interfering with an earlier or later link in the chain connecting organizing tissue or its precursors with the definitive axial system.

It is notable, however, that the viability effects of the same mutations do not show this quantitative type of relationship. Far from reinforcing each other in combination, they actually cancel each other. It is true throughout this series that individuals containing two *different* lethals are always viable. Such genotypes are indicated by asterisks in Table 2. Five of the six genotypes form balanced lethal lines when matings are made between animals of the same genotype. In the sixth combination (t^1t°) the male is sterile so no line can be established. Typical of the five is the behavior of the combination Tt° which constitutes a true breeding tailless line, as outlined in Table 3.

TABLE 3

	Tailless			Tailless
	Tt°			Tt°
Gametes	T			T
	t°			t°
Zygotes	TT	Tt°	$t^\circ T$	$t^\circ t^\circ$
	lethal 11 days	viable tailless		lethal 5-6 days

This breeding behavior shows not only that each of the mutant factors combined in the parent is lethal, but also that no crossing over occurs between the two factors, since two and only two types of gamete are formed by each parent. This type of evidence is supplemented by other breeding tests which have failed to disclose any crossing over between members of this series. We may therefore regard them as modifications of the same locus (alleles) or as otherwise prevented from crossing over.

The extreme case of a viable combination of two lethals is $t^\circ t^1$ in which each gene has a very early lethal effect. Since such combinations are viable it is unlikely that any of the *T* mutations are deficiencies, as in the Drosophila case investigated by Poulson. Their relations are more consonant with the assumption that they are point mutations.

This complementary action of factors which otherwise act as modifications of the same gene, can only mean that the effects on viability occur through different channels than the changes in morphological

pattern, and that although the latter exhibit quantitative relationships, the former cannot, since one appears to replace what is lacking in the other. It is assumed rather that the lethal effects are determined by processes which because of threshold phenomena yield "all-or-none" effects and differ in this way from allele to allele.

The separateness of morphological and viability effects is shown also by the normal survival of tissues from lethal embryos. Ephrussi has cultivated for several months tissues taken before death from *TT* embryos, showing that the cells are viable and that death is probably a consequence of faulty organization. This experiment, since mixed tissues were used, leaves open the question whether the lethal effect is localized in the derivatives of certain cells which fail to supply a proper stimulus upon which the organization of the whole depends. This hypothesis we hope soon to test by explantation methods. Dr. Gluecksohn has recently found that there is no umbilical circulation in the *TT* lethal embryos and they may die because of this fault. It is certain that they survive for several days in the uterus with very faulty growth, and other evidence indicates that lethal action is not merely an extension of morphological abnormality.

Thus we may answer the question propounded at the beginning of this section by saying that changes at the same genetic locus do seem to alter different developmental processes in different ways, in the sense that changes in morphological pattern show quantitative relatedness while changes in processes underlying viability behave as though qualitatively different.

Problems similar to those above have been studied in other ways and with other materials in the laboratory of Professor Baltzer at Berne. Baltzer (1940) has recently reviewed this work and compared it with results from the study of the mouse lethals. The point of departure of Baltzer and his collaborators was the discovery that certain hybrids and merogonic hybrids in Triton and between Triton and Salamandra regularly die in early stages characteristic for the given hybrid; and the experiments of the Berne laboratory have been directed mainly toward an analysis of the nature of the lethal effect and of the incompatibility between the nucleus of one species and the cytoplasm of another from which the lethal changes take origin. For our present purposes the facts of chief interest are those derived from a study of the growth potencies of different parts of lethal merogonic hybrid embryos. When, for example, a hybrid is produced by fertilizing an enucleated egg of *Triton alpestris* by a sperm of

Triton palmatus, the egg develops only to the stage of beginning closure of the neural tube, when it dies. *Triton taeniatus* cytoplasm with *cristatus* sperm produces an embryo which lives until about the eye vesicle stage and then invariably dies. These embryos are of course haploid but it has been shown that death is not due to haploidy, since intraspecific merogones develop normally. Although the whole embryo always dies, it has been shown by Hadorn and by Curry that certain of their organs will develop normally when transplanted into normal hosts of the maternal species. From the palmatus × cristatus embryo, heart, notochord, gut, epidermis, and myotomes all develop normally and become included in host structures. The head mesoderm and the neural tube of the same embryo on the other hand do not live when transplanted but die and degenerate.

Baltzer distinguishes four different stages of lethality, the early primary type in certain hybrids in which the head mesoderm becomes pycnotic even in a normal host; the secondary lethality of structures such as gills which depend upon the head mesoderm; and a later lethality of brain and spinal cord. Hadorn, by ingenious experiments in which parts of lethal merogones and of normal embryos were joined to form chimaeras showed that the abnormalities of brain and cord in the late lethal merogones were due to defective induction. Finally, a late functional lethality occurs when the muscles of the merogonic hybrid which develop normally fail to receive their innervation (through failure of neural tube and nerves) and subsequently degenerate.

Here then are departures from normal development, lethal in their effect and determined by the hereditary constitution of the nucleus in relation to the cytoplasm, which show something of the same chain-like relationship which we found among the changes at the *T* locus in the mouse. It is noteworthy that in each case, each nucleus of the embryo contains the lethal constitution, yet the abnormal processes begin only in certain regions, while others are affected only because their development depends upon the first. In the mouse the abnormal development and lethal effect occur during a stage of development which is specific for each mutation; and the same is true of the various constitutions which are lethal in the merogones.

In the lethal merogones it has been possible to test this localized origin of the abnormality experimentally and the results appear to me to be convincing.

We should now revert to the question raised in connection with the gradient hypothesis. If there the possibility had to be kept open that

below the "general" type of action might lie more localized or specific ones, so too, where the first disturbance found is localized, we have to ask, is this preceded by some more "general" cause which operates with especial severity upon this one region? There is no way at present to decide these questions and we cannot of course regard either "general" or "specific" as more than relative categories the usefulness of which depends on whether they lead us to make the observations and experiments necessary to disclose the facts. The truth is probably that the pattern of development itself is an alternation of general and localized factors which change places as causes and effects. Thus as in all problems of pattern we reach the ultimate question of the whole and the parts. What evidence we have from the pattern distortion brought about by hereditary changes testifies only to the fact that although the patterns of growth do maintain themselves in persistent wholes, their reality consists always in the existence of interdependent parts.

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NERVE PATTERNS: THE MECHANICS OF NERVE GROWTH[1]

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Compared with the steady flow of comparative-neurological research, analytical studies into the "how" and "why" of nerve pattern formation have remained a mere trickle. Whenever pressure for dynamic understanding of the development of the nervous system became irresistible, the comparative neurologist turned to speculation rather than to direct experimental attack. Kappers' "neurobiotaxis," Bok's stimulogenous fibrillation, even Child's gradient theory of nerve development, are noteworthy examples. There were sporadic attempts at analytical experimentation, but a deliberate drive did not really get under way until Harrison stepped in and gave the field an aim and a method; an aim, in that he broke down untractable generalities into tangible problems, and a method, in that he developed new techniques of transplantation and tissue culture for the solution of these problems.[2] His work, aptly extended by his students, notably Detwiler ('36), has been the greatest single step towards an analytical understanding of nerve development. Much of what I am to present today has had its roots in his work.

Harrison's experiments have settled a number of controversial issues concerning the elements of the nervous system, which we may list here as points of departure: (*a*) The neurone doctrine of nerve development was confirmed (Harrison '10): the nervous system arises from the differentiation of discrete cells. (*b*) The filamentous nerve processes, axons and dendrites, arise as extensions of the protoplasm of the nerve cell. (*c*) The cell strands enveloping the peripheral nerve fibers, or sheath cells, are later additions of central origin (Harrison '24). They play an accessory but not a formative rôle.

Nerve patterns, accordingly, do not emerge from a protoplasmic continuum, as was at times suggested (Hensen, Held), but are gradually built up in a true synthesis by the activities of individual neurones in constant interplay among each other and with their non-

[1]Experimental investigations reported in this paper have been aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[2]For the most recent review of Harrison's work, consult his *Croonian Lecture* (1935).

nervous environment. More specifically, the neurones multiply, differentiate, and aggregate in definite distributions, grow in definite directions, branch in a definite order, assemble into definite bundles, connect with definite organs, assume definite size relationships, and are enveloped by definite types of other, non-nervous, cells. Occurring according to a definite order in space and time, these processes produce the final nerve pattern of the adult, which is highly intricate. But the task of resolving it into simple developmental components is not as arduous as it may seem, for the following reasons: Firstly, we must keep in mind that the final pattern is of composite origin. That is to say, the pattern-determining factors, while varying from stage to stage, may at any one stage be relatively simple in themselves, the final complexity resulting from the superposition of the successive imprints left behind by each pattern in the form of definite cell groupings and fiber connections. A developed nervous system compares, in a sense, with a photographic plate upon which innumerable pictures have been exposed in succession. The second fact to be kept in mind is that the manifest features of nerve patterns are only in part active achievements of the nervous elements; partly they are the result of purely passive dislocations. Neuroblasts may actively aggregate by migration or be passively crowded together by the expansion of neighboring areas. The contorted course of a nerve fiber may indicate that the fiber took a crooked path in growing, but it may also be due to later distortions of an originally straight fiber.

Thus, resolved into its component steps, nerve development is really not so complicated that it would discourage analytical approach. With this in mind, let us now examine how far this approach has taken us.

The differentiation of a neurone proceeds roughly as follows: The neuroblast, after ceasing to divide, grows considerably in size. Next, a localized protrusion of protoplasm appears, destined to become the nerve sprout. Later similar sprouts appear on the opposite part of the surface. In the organism the first sprout usually develops into the axon, the other sprouts under considerable arborization into the dendrites. In nerve cells cultivated in vitro the distinction between axons and dendrites is frequently quite arbitrary. A nerve process elongates by amoeboid activity of its tip (Figure 3). This free end is in a constant state of unrest, sending out pseudopodia in various directions, of which one usually establishes itself, while the others are hauled in.[3] Then from the end of this pseudopodium new competing

[3]Instructive illustrations of the amoeboid growth of nerve fibers can be found in Speidel's work (1933).

feelers are stretched out into the surroundings, one of which again takes hold, and so the fiber extends farther and farther, until it is eventually arrested by connecting with other cells, by unsurmountable mechanical obstructions, or by nutritive exhaustion. In advancing, the amoeboid tip spins out the nerve fiber from the body of the nerve cell, which stays behind, anchored in the ganglionic tissue.

All nerve fibers arise in this way.[4] The first fibers to sprout out are fittingly called "pioneering" fibers. When they develop, the body is still of very small dimensions, and all tissues lie fairly close together. The greatest distances to be spanned by the pioneering fibers until they reach their destinations are at best of the order of the millimeter. Once connected with a peripheral tissue, epidermis cells or muscle buds, the roaming life of the pioneering fiber is over. Attached now at both ends, its further course is no longer one of its own choosing: the terminal tissues take it in tow and drag it along during their own extensive shifts.

The number of nerve fibers maturing early enough to act as pioneers is relatively small. Nerves at that stage consist only of a few fibers. New fibers are added as additional neuroblasts differentiate. Thus the fiber complement of a nerve is gradually built up. The road problem facing these later fiber generations is, however, quite different from that of the pioneering phase. Pioneering fibers must find their way in strange country and get orienting cues from a non-nervous environment. Subsequent fibers, on the other hand, need simply cling to the older ones to reach the same destinations. *Free* outgrowth has given way to growth by *application*.

Thus the establishment of a peripheral nerve involves three overlapping phases (Figure 1): First, the *free* outgrowth of a group of pioneering fibers through non-nervous surroundings. Second, the *bound* outgrowth of subsequent fiber generations along the line laid down by the pioneering fibers. And third, the *towing* process in which the nerve is drawn out by the growth and dislocations of its terminal tissues.

This developmental history of the neurone presents a number of clear-cut problems. Do the pioneering fibers move at random or do

[4]The idea of a peripheral rather than a central origin of nerve fibers has longest survived in the field of nerve regeneration. The autogenous regeneration of peripheral fiber fragments, advocated mainly by Apáthy and Bethe, has found adherents as recently as 1920 (Spielmeyer). However, all existing evidence is definitetly against any such view (Boeke, '35). It is true that abortive attempts at regeneration have been observed in distal nerve fragments in vitro (Levi, '34), but they are short-lived and of no practical significance.

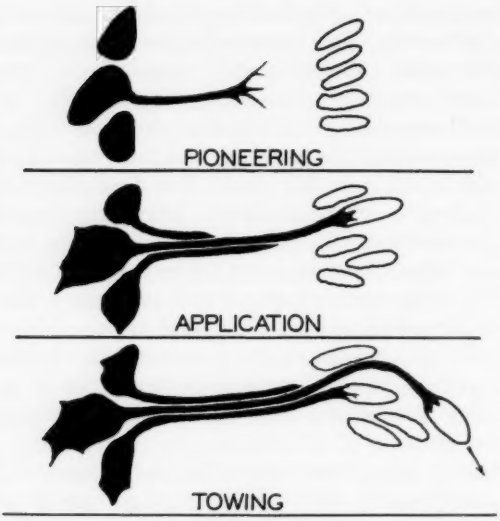

FIGURE 1

GROUP OF NEUROBLASTS IN THREE SUCCESSIVE STAGES

they follow definite courses? If directed in their outgrowth, what makes them find their way? Are they aimlessly following casual routes for better or worse, or are they aiming towards definite destinations? Do they connect with any terminal tissue, right or wrong, or does selectivity prevail in the process? Why do post-pioneering fibers follow the older fibers? And which ones do they follow?—And many more such questions.

Although the answers to some of them are beginning to emerge in their outlines, our analytical information is on the whole still very inadequate. Therefore, if I am to present to you a coherent story rather than loose scraps, I shall have to call rather liberally on hypotheses to do the cementing. Some gaps can be filled by borrowing analogies from related fields, others by thoughtful evaluation of purely descriptive data. But even so the picture will remain sketchy.

We exclude from consideration the factors that create the initial pattern of neuroblasts, as we find it in the early nervous system. The question of differentiation, i.e., of what makes embryonic cells different among one another, is one that cannot be solved for the nervous system separately. It is one of the most fundamental and

most obscure problems of all biology. We find neuroblasts of different potencies in different typical locations. These then transform into the different species of nerve cells. We do not even know whether they are already ear-marked for certain specializations when they are proliferated from the germinative (ependymal) layers, and then migrate each according to its type into a specified location, or whether presumptive nerve cells are still equivalent as they migrate into the various central districts, and then differentiate under the influence of local factors to which they become subjected in their final positions. We do not know, furthermore, what causes the proliferation of nerve cells to be confined to definite loci which shift from stage to stage and have been said to move in waves along the axis of the spinal cord (Coghill, '26, '36).

We, therefore, exclude from our discussions the problems presented by the differentiation of the early nervous system and begin at that later phase in which neuroblasts are already segregated, but nerve fibers have not yet appeared.

The first question of interest is, how does the neuroblast get polarized and oriented? It had been suggested by Child ('21) and Kappers ('17) that neuroblasts are polarized by the potential gradient of a surrounding electric field. Kappers viewed the case as one of direct galvanotropism, while Child suggested differential physiological response of the neuroblast at the anodal and cathodal poles. According to Kappers, the anodal outgrowth would become the axon, and the cathodal outgrowth the dendrite. All this is pure speculation. There simply are not sufficient data on hand to reach any decision. Péterfi and Williams ('33), who attempted to test the theory directly by placing neuroblasts into an electric field in vitro, failed to produce convincing evidence either way. One obvious difficulty of the whole theory lies in the frequent occurrence of tripolar and multipolar neuroblasts; it is hard to visualize how electric polarization could ever produce better than bipolar forms. The points at which the nerve processes emerge from the neuroblast are probably determined by some local changes in the cell surface. However, these changes need not be of electric origin. In fact, they need not even be of a single kind. In this connection Whitaker's ('40) splendid analysis of the polarization of the Fucus egg, reported at last year's symposium, deserves full attention as a possibly pertinent analogy. Similar experiments on neuroblasts are needed. Pending the outcome, we must suspend judgment.

This is not serious for our present purpose, because the further course of the nerve fiber does not directly depend upon the polarity and orientation of the nerve cell. Once the amoeboid tip of the fiber has left the cell body, it follows a course of its own. Our interest, thus, turns to the factors determining that course.

Several theories have been developed to account for the oriented outgrowth of nerve fibers. Strasser (1892) suggested the electric field as the orienting factor, and Child ('21) and Kappers ('17) considered the same potential gradients which had supposedly polarized the neuroblast as responsible for the further direction of the axonic outgrowth. However, aside from one positive claim by Ingvar ('20), which can be accounted for by deceptive experimental technique (P. Weiss, '34, p. 426), repeated attempts to demonstrate any orienting effect of the electric field on outgrowing nerve fibers, either in the embryo or in tissue culture, have consistently failed (P. Weiss '34; Karssen and Sager '34; Levi '34, p. 611; Williams '36; Gray '39). This negative evidence is strengthened by the fact that one frequently sees two nerve fibers grow out along a common path, but in opposite directions (Speidel '33). Such reciprocal fiber growth has recently been reproduced on a mass scale in experiments which will be reported below. Other experiments, also to be detailed later, have demonstrated that nerve fibers from the same source grow with equal ease in headward and tailward direction. Thus, whatever axial electrical polarity there may be in the body, has obviously no decisive effect on the direction of fiber growth. Consequently, the theory of a direct orientation of nerve fiber growth by potential gradients of an electric field finds no support in the facts.

Turning to the second group of theories, the chemical theories, or, more specifically, the concept of Cajal ('08), Forssman (1898, 1900), Tello ('23), that nerve orientation is produced by some sort of chemotropism, the existing evidence is overwhelmingly against them, too. Chemotropism implies a directive movement of an organism or part of an organism toward a distant source of chemical emanation. In order to operate, such a mechanism requires: (*a*) a constant source of chemical diffusion with a steady concentration gradient; (*b*) selective sensitivity of the growing part for that particular chemical; (*c*) an ability of the growing part to orient itself along the lines of the concentration gradient; and (*d*) a stagnant and homogeneous medium in which the concentration gradient can remain stationary during the whole process of oriented growth. These conditions are not realized

in the growing organism. There are, of course, numerous centers of chemical diffusion in the growing organism. However, nerve fibers do not follow the direction of concentration gradients, and even if they did, the body would not provide them with gradients of sufficient steadiness to serve as guides, as the agitation of the interstitial fluid between the tissues would blurr and distort the original concentration gradients beyond recognition. Furthermore, the fact of reciprocal fiber growth between two points is as fatal to the theory of chemotropism as it is to that of electrotropism, because it would mean that while one fiber runs up the concentration slope, the other one runs down. Also, in spite of several direct experimental attempts it has never been possible to demonstrate a direct attraction of nerve fibers towards a source of chemical emanation in vitro (P. Weiss, '34), not even towards degenerated nerve tissue, according to Cajal the most potent source of such action.[5]

Both the galvanotropical and chemotropical theories proceed from the assumption that the growing tips are oriented by factors acting from a distance. Nerve fibers are seen to *travel towards* certain localized regions and, hence, are assumed to have been *attracted by* those regions. For brevity, let us refer to this kind of orientation as a "distance effect." Now, before looking further into the problem, let us first check the solidity of its premises. In other words, are nerve fibers really moving towards definite destinations, or are they merely running along some preformed traffic routes?

The growth of the segmental nerves along the boundaries of the myotomes, the growth of the lateral line nerve down a longitudinal groove along the trunk musculature, the coursing of the limb nerves along the main blood vessels, and similar facts, would seem to make nerve orientation a matter of the road rather than of the destination. His (1887) has stressed the predilection of nerves for mechanical roadbeds, and Vanlair (1885) has gone so far as to claim that nerves simply take the way of least resistance. Exceptions, in which nerves seem to have gone out of their way, so to speak, in order to reach distant organs, such as the long backward loop of the vagus from the

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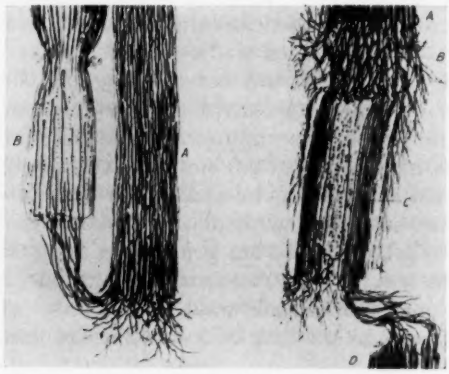
[5]Centanni ('14) once reported "nerve growth" in vitro as oriented towards explanted tissue fragments of supposed chemotactic activity. His statement, however, contains a gross misrepresentation. inasmuch as his "nerve growth" came from isolated fragments of peripheral nerves, in which, as we know, all nerve fibers degenerate rather than regenerate. The so-called "nerve growth," therefore, can have reference only to emigration of spindle cells from the fragments, which has no bearing on the problem of nerve growth at all.

head to heart and intestine, or the long forward swing of the lingual nerves into the tongue are deceptive, in that these courses reflect extensive shifts of the terminal musculature of these nerves in the towing phase, rather than directive free outgrowth of the nerves. So, one cannot dismiss the possibility that nerves connect with their proper peripheral areas simply because these areas happen to lie at the end of those lines which the nerves find open when they start to grow out. And the problem of whether nerves are destination-bound at all, is a real one.

Experimental evidence on the point is meager but suggestive. Cajal ('08) and Forssman (1898, 1900) have tried to prove under a wide variety of conditions that nerve fibers in the process of regeneration grow actually toward a goal rather than merely along a path. Let me cite one of their classical experiments, exemplary for many others. When a nerve is cut, the distal fragment transforms into a non-nervous protoplasmic cord which persists as such, while the proximal fragment gives rise to abundant outgrowth of new fibers (cf. Boeke, '35). Whether the two stumps are directly apposed or whether they are dislocated, in either case a large number of the new sprouts is finally found trapped inside the old degenerated tubes (Figure 2). The orientation of the regenerating fibers is by no means strict. Numerous



A B

FIGURE 2

NERVE REGENERATION INTO DISLOCATED PERIPHERAL STUMPS (AFTER CAJAL)

A, Nerve fibers from a severed nerve (*A*) have partly found their way into a degenerated nerve fragment lying some distance behind the cut surface.

B, Nerve fibers from the proximal nerve stump, *A*, after pervading the scar, *B*, have partly entered, partly by-passed, the degenerated nerve segment, *C*; part of the fibers emerging from *C* have traversed the gap to the dislocated fragment *D* and grown into it.

fibers can be seen to have strayed about at random. Nevertheless, a large proportion has reached the peripheral nerve stump. In order to do this, they had to take a somewhat contorted course. Doubtless, the degenerated peripheral stump has exerted some potent influence on the pattern of regeneration.

However, in specifying this influence, we must be aware of two possibilities: One is, that the sprouting fibers were attracted by some factor emanating directly from the open end of the distal fragment. This is the interpretation given to the results by both Cajal and Forssman. In view of experiments which we shall take up in a moment, however, a second possibility seems more likely.

Suppose, the fibers stray out at random. Some strike the peripheral stump by accident. This puts an end to their period of vagrancy. Then something happens to them: As a result of their having become settled, their condition changes; changes in a fashion that will render them sticky or otherwise attractive for other nerve fibers growing out later. In this way, more and more fibers will get trapped along this preferential course. Thus, what originally was accidental encounter, becomes gradually a systematic pursuit. The successful bridging of the gap would be a matter not so much of directive fiber outgrowth, as of the fact that an accidentally successful nerve sprout becomes the center for the building up of a nerve cable. We will call this process *"selective fasciculation"* and return to it in greater detail presently. For the moment, we merely mention it to show that Cajal's experiments do not necessarily prove the case of destination-bound nerve orientation.

Experiments on the embryo, reported by Detwiler (reviewed '36), seem to be more conclusive. The nerves of a vertebrate limb are of plurisegmental origin and converge upon the limb base, where they intertwine in a plexus. When a limb bud is transplanted to a more anterior or posterior position, the innervating centers shift likewise, but less, and there is a certain tendency of nerves to slant more anteriorly in the former, and more posteriorly in the latter case. In spite of great individual variations, limb nerves, as a rule, show a more headward slant if the limb bud was transplanted more anteriorly, and a more tailward slant in the case of a posterior shift. Since the limb plexus converges likewise upon a transplanted nasal organ (Detwiler '28) a tail, or an eye (Detwiler and Van Dyke '34), the attraction, if such it is, is certainly not very specific. Nevertheless, a

general diversion of nerves toward growing organs seems to be indicated.[6]

Unfortunately, only the end stages of this process are known, and since secondary events, such as extensive shifts of musculature, plexus formation and, possibly, selective fasciculation, have distorted the original situation as it existed at the moment of the outgrowth of the nerves, it remains unknown just how potent the supposed attraction of embryonic nerves by growing organs is. However, since Burr ('32) has shown that nerves growing out from transplanted nasal organs are likewise showing definite preferences as to the point of ingrowth into the embryonic brain, and since, furthermore, Coghill ('29) has come to the conclusion that in the development of the brain nerve fiber tracts tend to grow towards centers of simultaneous differentiation, we may take it for granted, pending proof to the contrary, that growing regions of the body exert a general influence, which in purely descriptive language may be termed "directional attraction."

The acceptance of this fact puts us into a real dilemma. We admit that nerves can be attracted towards distant organs, and at the same time deny the validity of the best known mechanisms of distance action—galvanotropism and chemotropism. The alternative to distance action is what, with a general term, we may call *"contact action."* All present evidence points to the fact that nerve fibers are conducted on their way solely by "contact action," and in order to extricate ourselves from our dilemma, we would have to prove that distance effects can actually be resolved into contact actions.

This we can prove. But before doing so, we must give some closer attention to the mode of progression of the growing nerve fiber. This takes us into a much wider biological field—the problem of amoeboid movement. Amoeboid movement is produced by the protrusion of lobular or filamentous processes from the protoplasmic surface, so-called pseudopodia, which take hold on the substratum and cause the cell content to stream after. Some cells can project pseudopodia into a homogeneous liquid. Nerve fibers cannot. The nerve protoplasm needs a surface or interface along which to extend. Perhaps this need is imposed by the great length of the fibers. Since the tremen-

[6]Cases of extensive nerve detours, as if aiming to reach nerve-free limbs, described by Hamburger ('29), owe much of their impressiveness to the secondary distortion which the nerve courses have suffered during the towing phase. In view of the close apposition of the young limb buds and the closeness of their respective nerve fiber pools, contralateral innervation occurs under infinitely simpler conditions than the final developed product would make one suspect.

dous expansion of relative surface in elongation requires a large output of energy, it is possible that the task would go beyond the capacity of metabolic energy production by the cell, so that external physical structures would have to be called on for support.

However, whatever the reasons, nerve fibers positively do not grow when suspended in a homogeneous liquid medium. When cultured in liquid media in vitro, they cling to the surface of the coverslip or to the surface of the drop (Lewis '12, Levi '34). The occasional observation of nerve fibers inside the spinal canal, which is filled with cerebro-spinal fluid, is no exception, as such nerve fibers have most likely grown along the inner wall of the tube and merely become swept into the liquid in the act of fixation. Let us repeat, therefore, the pseudopodia of the growing tip of a nerve fiber extend along phase boundaries, and only along such.

These phase boundaries need not be of the gross macroscopic kind, such as coverslip–liquid, or liquid–air. In fact, they rarely are. Much more commonly they are of microscopic or submicroscopic order. In the embryo the growth of a nerve fiber along minute microscopic threads has been repeatedly described (cf. Held '09). But since, what in a microscopic preparation appears as a fibril, is often an artifact, resulting from the clotting together of identically oriented ultramicroscopic structures, it is most likely that even in these cases nerve growth in the living occurred along some ultramicroscopic rather than microscopic phase boundaries. The same is true of tissue culture in a blood plasma clot. The plasma coagulum is essentially a spongy gel consisting of a more solid skeleton of fibrin micellae in various degrees of aggregation, imbibed with a continuous phase of blood serum. Interfaces between fibrin aggregates and serum thus are present everywhere in the clot, and they furnish the necessary substratum on which the pseudopodia of growing nerves may take hold.

The question has been raised of how a microscopic body, such as a nerve fiber, can follow an ultramicroscopic interface, which is of lower order of magnitude. The answer is simply that it is not the microscopically visible nerve fiber, which plays the active part in the creeping process, but its delicate terminal pseudopodia, which may very well taper to submicroscopic dimensions.[7] We know that what

[7]Grossfeld ('34) has overlooked this fact when taking issue with the explanation of oriented fibrocyte growth in terms of the ultrastructure of the medium. Obviously, what we have stated here about nerve fibers, applies equally well to fibrocytes, which likewise advance by means of fine filamentous pseudopodia.

under the microscope appears as the tip of the pseudopodium of a nerve fiber, is not its real end; Giuseppe Levi was able to trace the filaments far beyond that point in dark-field illumination, and we have, of course, no assurance that they may not even extend beyond the limit of dark-field visibility. This makes the pseudopodial tips and the interfaces to which they apply themselves appear as of commensurable dimensions. Once established, the pseudopodium is soon enlarged to microscopic dimensions by the inflow of protoplasm, while new pseudopodia issue from its extreme tip and penetrate into the medium (Figure 3).

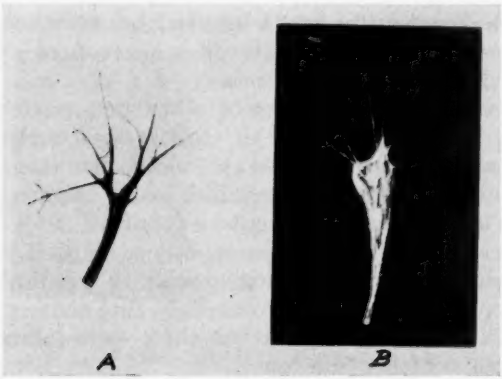


FIGURE 3

PSEUDOPODIAL RAMIFICATION OF THE GROWING TIP OF A NERVE FIBER CULTIVATED IN VITRO (AFTER LEVI)

A, Fixed and stained preparation.—X 950. *B*, Dark-field view of living specimen.

The tendency of organisms to cling, as they grow, to solid structures, is commonly referred to as *stereotropism*. This is a naturalistic term. It classifies the phenomenon, but reveals nothing about its nature. The movement of a nerve fiber falls into this class. Our analytical insight into the phenomenon does not seem to have advanced far beyond where it stood when Quincke, in 1888, explained protoplasmic movement as the work of surface forces, comparing it with the spreading of oil on a water-air interface. It is to be hoped that such studies of the mechanism of amoeboid motion as have been carried out by Mast ('31) on amoebae, by Lewis ('39) on macrophages, and by Fauré-Frémiet on amoebocytes, may lead to informa-

tion which can be applied to the nerve fiber. For the time being, however, our ideas about the process are still highly speculative.

We are probably safe in saying that interfacial tensions between the nerve fiber protoplasm and the micellar aggregates along which it extends are at work, but just how they operate is still obscure. Of the whole protoplasmic system only that fraction counts which takes part in the surface and is in actual contact with the medium. Reversible gelation of the cortical protoplasm endowing the surface film with adhesiveness, as suggested by Lewis, may be an essential element of the mechanism. But this "adhesiveness" itself can bear further analysis. Are we simply dealing with a purely colloid-mechanical phenomenon, or might we not have to postulate molecular affinities of greater specificity? I have observed some nerve fibers in vitro following a pathway which was wholly ignored by others. This and many similar experiences suggest selective "adhesivity" rather than a common kind of stickiness.

In an unoriented gel of isotropic constitution phase boundaries extend from any one point in several directions. This favors the simultaneous protrusion of several pseudopodia along different lines. (Figure 3). Which one of them is to endure, depends probably largely on local accidents. But it is reasonable to assume that the inertia of the centrifugal protoplasmic flow in the growing sprout will generally enhance the repletion of those pseudopodia that lie more or less in direct line of the axis of the sprout. We have little accurate data about protoplasmic movement inside the fiber, but Levi ('34) has observed some agitation, and Speidel's films show some kind of peristaltic motion. Once a pseudopodium has established itself and caused the mass of protoplasm to flow into it, the drain thereby exerted on the other pseudopodia produces their automatic withdrawal. Only if two pseudopodia happen to be of approximately equal strength, they may divide the protoplasmic influx between each other and thus initiate a terminal branching of the fiber (Figure 11). We shall return to this point. In a medium lacking definite orientation, the nerve processes will thus follow random courses. This is actually realized in ordinary tissue cultures as well as in many parts of the central neuropil.

However, from what we have said, it can be anticipated that if nerve fibers are confronted with a medium in which the interfaces follow a common instead of random direction, their growth, bound to retrace those interfaces, would become likewise oriented, just like

vines, growing along parallel stakes. This expectation has been borne out by tissue culture experiments. Since these have been reported at length (P. Weiss '34), we need not go into details. When nerve fibers are made to grow out in a blood plasma clot which had been stretched or otherwise put under tension, the course of the fibers follows the pattern of tension. The tension has served to turn the polarized fibrin micellae into a nearly parallel orientation, and consequently all pseudopodia were likewise drawn into a nearly parallel course.[8] To obtain the effect, the tension need not be exerted from without. Living

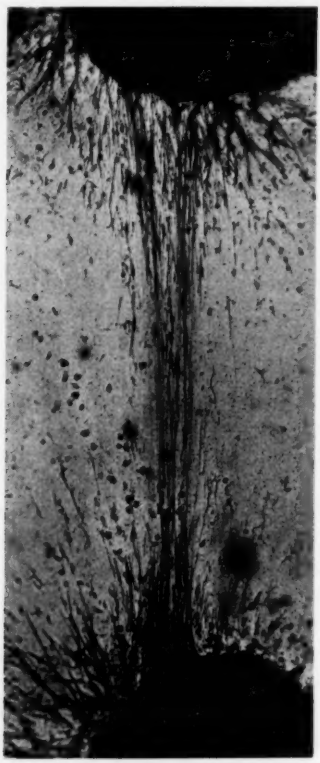


FIGURE 4

ORIENTED NERVE GROWTH CONNECTING TWO SPINAL GANGLIA CULTIVATED IN A THIN MEMBRANE OF BLOOD PLASMA IN VITRO (x 48, P. WEISS, '34).

[8]For illustrations of the effect, see Figures 1-4 in P. Weiss, '34.

proliferating tissue by itself has a contracting effect on the surrounding medium and thereby produces tension. Two such centers of contraction in a common medium automatically produce a pathway of tensionally oriented micellae, which, if nerve cells are present in the centers, becomes soon populated by parallel nerve fibers growing out from both ends in opposite directions (Figure 4). Incidentally, this is another example of reciprocal fiber growth, defying galvanotropic and chemotropic interpretations.

The fact that in these experiments the agent to produce micellar orientation happened to be tension, is irrelevant. For any ultrastructural orientation of the medium, no matter how attained, may have the same effect on the nerve fibers. Convection of liquids through the medium can produce such orientation, and vectorial aggregation of micellae may even occur spontaneously. On the whole, it is possible to explain the oriented outgrowth of individual nerve fibers by taking into account the colloid-physical structure of the medium, and the tendency of the nerve protoplasm to comply with it. The stereotropism of nerve fibers with regard to solid microscopic structures, as assumed by His, and emphasized by Harrison ('14), seems to be only a special case of the more general principle of contact guidance of the nerve tip by the ultra-structure of the medium. To call this contact principle "mechanical," misses the point, the more so as we do not yet know of how much selectivity the adhesiveness to the contact structure admits. It is quite conceivable that differential chemical impregnation endows certain ultra-structures with greater affinity to nerve pseudopodia than others (see below).

Nerve fiber orientation was thus explained, at least in principle. The attraction of nerves towards growing organs, nerve branching, the formation of nerve plexus, and the reciprocal outgrowth of nerves between simultaneously differentiating centers, could be reproduced in vitro. More than that, the experiments have definitely proven that nerve fibers are actually guided by contact and not attracted by agents operating from a distance. So, when nerve fibers are seen to move towards a remote destination, one may take it for granted that that goal was instrumental in laying down an oriented pathway in the medium, rather than acting on the fibers directly. Established in tissue culture, all this certainly holds true for nerves in vitro. But does it likewise apply to the living organism? And if so, does it help to explain the nerve patterns as they actually arise in the course of development?

As I have pointed out on earlier occasions ('33, '34), the conditions between tissue culture and organism are fundamentally comparable in many respects. However, the actual testing ground is the organism, and this test has thus far remained wanting. Moreover, there are certain features of nerve growth in the organism which cannot be repeated in tissue culture. There was urgent need to clarify the situation in the organism directly. During the last few years I have tried to approach this task by devising methods specially adapted to the problem. The problem was to let nerve growth occur under conditions infinitely simpler and more transparent than those under which it normally occurs and whose complexity defies direct analysis, but still in the organism.

Two different techniques were found to be successful. In both amphibian larvae are used. The first consists of producing an extensive bed of simple granulation tissue and allowing nerves to regenerate into it under various controllable conditions. Locations chosen for the purpose were the *skull cavity,* from which the brain down to the anterior end of the medulla oblongata had been removed, and the *orbit of the eye* after enucleation of the eye ball (see Figure 9, *A, C*).

Even large tadpoles survive the removal of brain of the indicated extent for six weeks or more. The skull cavity fills with a soft, loose connective tissue, which provides our testing ground for nerve growth. This nerve growth comes from four sources, the two stumps of the olfactory nerves and the two optic nerve stumps, all four belonging to nerves whose cells lie in the periphery and grow centripetally (Figure 9, *C*). Similarly, after enucleating the eye under certain precautions, the orbital space becomes filled with a simple vascularized connective tissue, and a source for nerve regeneration is made available by cutting the trigeminal nerve, which runs along the inner wall of the orbit (Figure 9, *A*). These experiments have furnished instructive information concerning the relation between nerve growth and connective tissue orientation, relations between nerves and capillaries, the non-existence of any orientation of nerve fiber growth with regard to the general body axis, as well as the non-selectivity of nerve associations (see below), but lack of space prevents us from discussing these points in greater detail at this time.

The second method may be called *deplantation.* It takes advantage of the fact that the amphibian larva contains one extensive and readily accessible tissue which in itself is so elementary that it com-

pares in simplicity almost with a tissue culture, namely, the gelatinous connective tissue of the fin. It consists (Studnicka, '38) of a gelatinous keel-shaped mass extending over most of the dorsal midline, sparsely settled with cells, and pervaded by a moderate amount of capillaries and sensory nerve fibers. Into this tissue we transplant large fragments of developed spinal cord or brain from another larva (P. Weiss, '40, '41, '41*a*). These fragments are to furnish outgrowing nerves. The transplanted mass undergoes partial resorption, but what is left is sufficient to serve as a potent nerve source. This method has the advantage that other organs can be transplanted into the vicinity of the deplanted center so that the effects of different organs on nerve growth can be analyzed, and, in fact, directly observed, because the fin tissue is sufficiently transparent to permit direct observation of the gross events.

Let us see what happens when a brain fragment and a limb are incorporated in the fin at some distance from each other. (Figure 5).

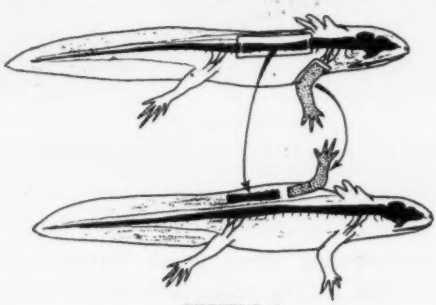


FIGURE 5

DIAGRAM OF DEPLANTATION EXPERIMENT

A portion of the spinal cord and a fore limb are excised from the donor animal (top) and implanted at some distance from each other in the dorsal fin of the host (bottom).

The result is quite impressive: Within two to four weeks after the operation a strong nerve cable forms between brain graft and limb (Figure 6). Inside the limbs these nerves follow the regular nerve channels and make functional connections with the musculature. Innervation completed, the whole grafted complex begins to exhibit spontaneous functional activity which is highly interesting from a physiological standpoint, but which to discuss is beyond the scope of this paper.[9]

Now, in these experiments we have witnessed nerve formation in

[9]The functional results have been summarized in P. Weiss, '41.

an almost diagrammatically simple form. Given: an isolated nerve center and an isolated limb in a common gelatinous matrix. Result: a direct nerve connection. That the limb is the actual objective of the outgrowth, is proved by various facts. If no limb is transplanted, only stray fibers leave the brain fragment, but no nerve cables form. Depending on where the limb was inserted, whether behind or in front or on top of the grafted nerve center, the nerve cable grows posteriorly or anteriorly or dorsad, which again proves that the polarity of the body is immaterial in nerve orientation. If two limbs are transplanted, one anteriorly and the other posteriorly, nerve connections form in both directions. Histologically, these nerve cables

FIGURE 6

LATERAL VIEW OF THE TRANSPARENT FIN OF AN EXPERIMENTAL ANIMAL CONTAINING DEPLANT OF SPINAL CORD (*G*) AND GRAFTED LIMB (*L*): A STRONG NERVE CAN BE SEEN TO HAVE GROWN FROM *G* TO *L*

(13 weeks p. op.—cca 6 x.)

consist of non-anastomosing fibers, which, interestingly enough, are devoid of sheaths and sheath cells, just like a central fiber tract (Figure 7). It is noteworthy that they are fully functional, proving the physiological adequacy of naked peripheral nerves as conductors.

As for the analysis of the phenomenon, one technical detail must be taken into consideration. In order to introduce the deplants, a longitudinal channel was made in the fin gel; the brain fragment was deposited in the distal part of this pocket, and the limb was usually inserted into its orifice. Residues of this channel would present a road of least resistance between the deplant and the limb graft and favor nerve growth between the two structures. As you will see, this explains part but not all of the phenomenon. For, even if the limb and the brain grafts are introduced into separate pockets, without open communication, nerve connections between the two are still established. These differ, however, from the channel connections in one notable respect. Instead of a single straight nerve

cable, there are usually several nerves, and instead of being straight, their courses are somewhat crooked.

Nerve cables, though of smaller size, may form also between the deplant and underlying trunk musculature. Here, too, the presence of a connecting channel facilitates the connection, but is by no means essential.

If we focus, for the moment, on the cases without common channel, we realize from the crooked course of the nerve connections that they have not come about by directive outgrowth, but rather by differential fasciculation. More specifically, this is the sequence of events. Nerve fibers, singly or in small groups, radiate from the deplant in all directions and stray through the surrounding fin tissue. Some

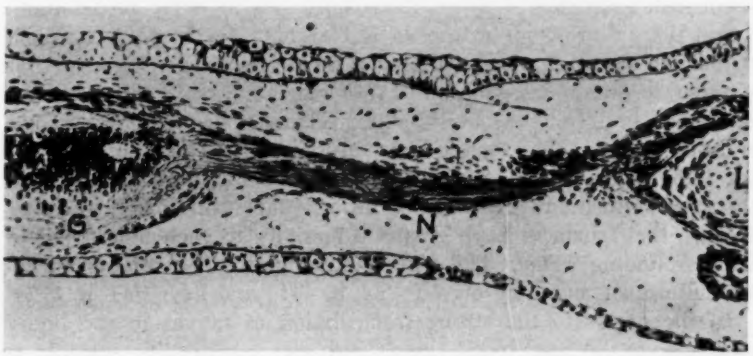


FIGURE 7

FRONTAL SECTION THROUGH FIN OF EXPERIMENTAL ANIMAL CONTAINING DEPLANT OF SPINAL CORD (*G*), TRANSPLANTED LIMB (*L*), AND CONNECTING NERVE CABLE (*N*) (18 days p. op.—x 72.) Impregnated according to *Bodian*.

have reached the grafted limb, others have not. Only those nerves, however, which have entered the limb, have subsequently become filled up into a sizable bundle. This leaves only one interpretation. Obviously, those pioneering fibers which had accidently struck the limb, had thereby acquired some contact property which made their surface sticky, or otherwise a pathway of preferential application, for other fibers growing out subsequently. This is precisely what I had suggested before as an alternative to Cajal's chemotropism in the regeneration of nerves; only here we have more direct evidence. Just how the fasciculation of "successful" fiber courses comes about, has not yet been decided. Nor do we know whether the successful fiber

owes its rallying power merely to the fact that it has come to a halt as a result of terminal connection, or rather to some specific chemical impregnation which it may have received from its new end organ.

The straightness and singleness of the nerve cable in the channel experiments is easily accounted for as a special case of selective fasciculation. For the channel scar establishes a straight connective tissue bridge between the limb and the deplant, along which pioneering fibers are actually guided toward the limb, as in our tissue culture experiments. For the rest, the gradual building up of the cable occurs according to the same rule as in the other series, in which the encounter with the limb is purely a matter of chance.

A deeper analysis of the change, presumably in the surface, which distinguishes successful fibers from unsuccessful ones, is definitely within the limits of our methods. But nothing has as yet been done in that direction. One point, however, seems to emerge. That is, that in order to obtain fasciculation, the immediate vicinity of the nerve fiber must be in a fairly liquid state. This can be observed directly in tissue culture. While nerve fibers inside the solid plasma clot grow out individually, they anastomose and associate easily in liquefied parts of the medium. Nerve fibers in a liquid medium stick together because their surfaces have greater adhesivity to each other than to the surrounding serum. This rôle of liquidity for fasciculation in tissue culture was noted by myself ('34, p. 441) and Levi ('41, p. 177).

In the organism the strong fasciculation of nerves in the liquid spaces along the large blood vessels furnishes a suggestive example of the same situation. We can almost generalize and say: Inside the solid tissues—epithelium, muscles, central gray, and so forth—nerve processes tend to take individual courses. In between these tissues, however, where the fibers pass through liquid-filled spaces, they are grouped into nerves. Now, if liquidity of the medium is a prerequisite for fasciculation, part of the task of a successful pioneering fiber may consist of liquefying its immediate surroundings by proteolysis.

Indications of facultative proteolysis by nerve fibers have been observed in tissue culture.[10] Nerve fibers in old cultures, that is those

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[10]This remark does not refer to the proteolysis of the plasma clot commonly observed in brain cultures (cf. Olivo '28, Levi '34, P. Weiss '34), which has been shown (P. Weiss, '34*a*) to be due to the secretory action of ependymal *cells* (and which, incidentally, has been wrongly interpreted by Levi, '41, as plasma syneresis). We are referring here to proteolytic effects of the *nerve processes*. A few examples were observed during earlier work (P. Weiss, '34, p. 434). More extensive evidence has been obtained since, but has not yet been published.

in which growth has essentially come to a standstill, frequently begin to dilute the surrounding plasma medium and, at the same time, combine into bundles (Figure 8). In favorable cases, the formation

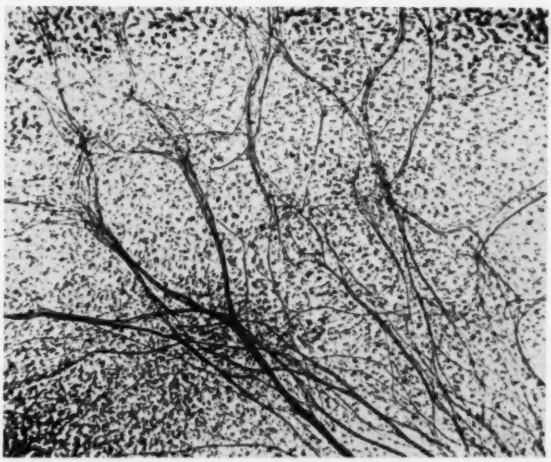

FIGURE 8

PERIPHERAL GROWTH ZONE OF TISSUE CULTURE OF SPINAL GANGLION (CHICK EMBRYO) AFTER SEVEN DAYS' CULTIVATION IN PLASMA CLOT

Extensive bundling of nerve fibers. Impregnated according to *Bodian's* method. x 56.

of blisters in the vicinity of a nerve can be immediately observed. However, whether these cases have a real bearing on the mechanism of fasciculation in the body, remains to be seen.

But whatever the details may be, nerve fibers which have "arrived" somehow bear the stamp of success on their exterior, which induces other nerve fibers to follow them, while unsuccessful ones, lacking similar appeal, usually remain unattended. In the light of these results, the significance of primarily oriented fiber outgrowth for the development of nerve patterns seems to be greatly reduced. The problem obviously exists only during the pioneering phase of early fiber growth and regeneration; during that phase it is apparently well covered by the principle of *ultrastructural orientation*. But from there on all further development of peripheral nerves is a matter of *selective fasciculation*.

This immediately raises two questions: Firstly, the question of what degree of selectivity there is in the application of one nerve fiber to

another. And second, the question of what kind of numerical control limits the size of a nerve cable to its normal proportions.

Let us consider the problem of selectivity first. Selectivity may assume two forms: selectivity of pathway and selectivity of termination. Therefore: can nerve fibers select their courses, and can they choose the tissues with which to connect? To take up the latter point first, the notion that nerve fibers from a given source would possess a prerogative on a corresponding pre-assigned peripheral organ, is wholly untenable. Any number of cross connections between nerves and terminal areas strange to them have been successfully effected.

A brief list may indicate the range of non-selectivity. First, with regard to regeneration: Motor fibers can connect with sensory organs, and sensory fibers can form terminations in muscles (Boeke, '17). It has been shown that such connections may even be physiologically adequate: a dorsal spinal root (P. Weiss, '35) or a sensory nerve (P. Weiss '34b) forced upon a muscle can mediate its contraction. Any motor nerve will connect with any muscle. It even connects with spinal cord when inserted into it (P. Weiss '32). In the deplantation experiments, just discussed, innervation of limb and trunk muscles was obtained from all spinal cord, medulla oblongata, midbrain, and forebrain, all of which had reached functional differentiation at the time of deplantation (P. Weiss, '41a). Turning to the embryonic phase, cranial nerves were shown to penetrate limbs (Harrison, '07; Braus, '05, Detwiler, '30; Nicholas, '33) or trunk muscles (Hoadley, '25), grafted within their domain; and in fact, central fiber tracts can do the same (Nicholas '29, '30).

I can supplement this array further with an example from our recent experiments on nerve growth in the cleared orbital or cranial cavities, which I mentioned before. In the empty skull cavity the regenerating fibers of the optic and olfactory nerves pervade the granulation tissue in all directions, forming an unorganized neuroma. But in most cases there were also strong fascicular connections between at least some of the nerve stumps. Quite aside from their bearing on the problem of fasciculation, they interest us here for the weird combinations they have produced. For instance, the optic and the olfactory nerves had met head-on, merged into a common cord, whereupon the optic fibers have travelled on towards the nose, and the olfactory fibers reciprocally towards the eye (Figure 9, *D*). Their ultimate fate has not yet been studied, but the gross fact is clear. Similarly, in the enucleated orbit regenerating fibers from the severed

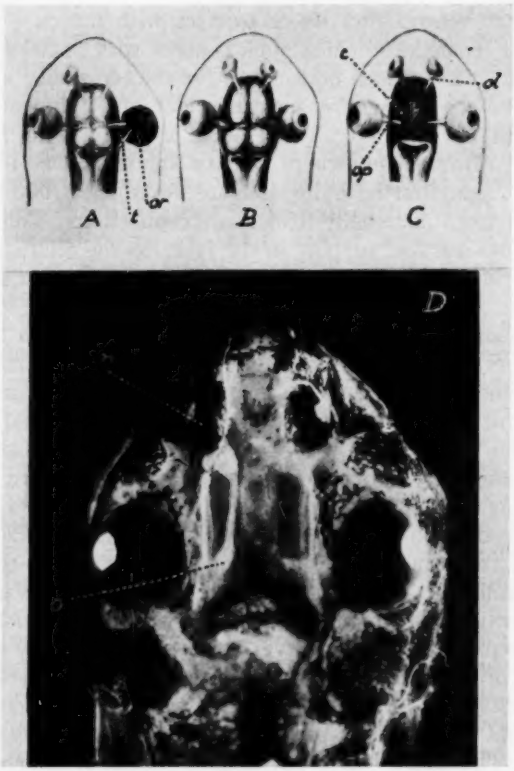

FIGURE 9

METHOD OF STUDYING NERVE GROWTH IN EXPERIMENTALLY PREPARED CAVITIES AS BEDS

A, Orbital bed (*or*), cleared by enucleation of bulb, with trigeminal nerve (*t*) as fiber source.

B, View of normal brain of frog tadpole after dorsal opening of skull.

C, Cranial bed (*c*), cleared by removal of all brain parts anterior to cerebellar lamina, with olfactory (*ol*) and optic (*op*) nerves as fiber sources.

D, Experimental animal, operated according to *C*, showing fusion of left optic (*o*) and olfactory nerve (coming from nose, *n*) into common cable.

trigeminal nerve have penetrated into the central stump of the deserted optic nerve and grown back into the brain.

In spite of this demonstrated non-selectivity of terminal connections, the fact remains that in normal development highly specific relations are somehow achieved. The simplest expression of these is

the fact that ventral root fibers connect with muscles, while fibers growing out from the spinal ganglia connect with sensory end organs, mostly in the skin. Since both travel for some distance along a common road before segregating into muscular and cutaneous branches, they present us with a really puzzling problem. This problem has not yet been brought anywhere near its solution. One might submit that there might first be indiscriminate outgrowth, followed later by the reduction of functionally inadequate connections. This contention, however, does not find support in the facts, as the relations are established very early, long before there can be any question of functional effects.

Mr. Taylor, of our laboratory, has made a thorough investigation of the innervation of hind limbs of frog larvae, which had developed in the absence of either their sensory or their motor nerve sources. Purely motor innervation was obtained by the removal of the sensory ganglia prior to the outgrowth of the limbs, and purely sensory innervation by the early extirpation of the whole spinal cord of the limb area, leaving the ganglia undisturbed. Even the very early limb bud, long before differentiating muscle and skeleton, is already pervaded by sprouts from the nerves which had been waiting at the limb base in large numbers. Later, as the limb elongates, the various nerves can be identified. In animals with purely motor centers most fibers are then found in muscular nerves, while purely sensory sources have sent most of their fibers along cutaneous pathways. The separation is not strict. Motor fibers get into the skin and cutaneous fibers into muscles, but a statistical predilection of each kind is nevertheless indicated. This bears out an earlier observation of Hamburger ('29). The fact seems clear, its explanation less so. We are still collecting evidence, and I do not want to be too positive. But one thing seems to emerge: If there is selectivity in these early stages, it seems to refer to the pathway rather than to the terminal areas. Sensory pioneering fibers would show affinity to a cutaneous nerve path rather than to skin; motor fibers to muscular nerve paths rather than to muscle. These paths might be viewed, in line with our earlier discussion as ultrastructures with differential chemical impregnation. Sensory and motor nerve fibers would have to react differentially to the two types of structure. In assuming this, we are on firmer ground. We know from anatomical and physiological experiences that motor and sensory fibers are constitutionally different. This differential might very well predispose them for different contact affinities. Of course, once the

pioneering fibers have been sorted into their proper channels, the rest may be merely a matter of selective fasciculation: Guided by surface affinities, sensory and motor fibers would apply themselves each to the corresponding type.

The fact of surface affinities among identical kinds of nerve fibers seems to be well supported. For what else can it mean when the neurologist describes the fibers which mediate special sensations, such as pain, proprioception, cutaneous sensation, and so forth, as running in their intraspinal course as separate fiber bundles? Or the fact, that even in a peripheral nerve the sensory and the motor fibers hold pretty much to themselves, although imbedded in a common nerve tract? Each bundle contains fibers of a wide range of ages, which have developed in succession; if they lie together, this must mean that the later ones have grown out along the path of the earlier ones. Oppenheimer ('41) has recently described interesting observations on the course of supernumerary Mauthner's fibers in the fish brain. The extra fibers, easily recognized by their size, have shown a marked tendency to follow the course of the original Mauthner's fiber. This is strong evidence of selective surface application. Thus, it may become necessary to concede to nerve fibers the attribute not only of being just adhesive, but of *selective adhesiveness*. This affinity would presumably be obliterated by the appearance of a myelin sheath.

The problem of selective fasciculation brings up a question of much wider biological significance, namely, that of tissue affinities in general. The intimate fusion or adhesion between tissues of different origin presupposes a compatibility between the contact surfaces which thus far has not found sufficient elucidation. Whether two protoplasmic systems can adhere to each other, depends primarily on some contact affinity which must hold at least until mechanical ties, such as basement membranes, or other fiber structures, have cemented the surfaces. Holtfreter ('39) has reported some highly instructive observations on how this contact affinity among different tissues changes in the course of ontogeny; how tissues which at one time have adhered, later separate off, as their biochemical divergence becomes greater.

I am inclined to regard this merely as one special case of a very general biophysical problem, which finds its expression in such diverse phenomena as the selective fusion of protozoan pseudopodia, depending on whether they belong to the same animal or to different animals; the fusion of individualized cells with a common syncytium and later

re-formation of individual cells within the syncytium, depending probably on variations of biochemical constitution of the respective nuclear territories (see P. Weiss, '40*a*); the different rate and ease with which grafts can be incorporated depending on their character and orientation; the differential agglutination of sperm in the presence of different types of eggs; and many other similar phenomena. We may view all these phenomena in a common light, as follows (Figure 10).

Tissue affinity may be based on the presence in the contact surface of identical (*B, C*) or complementary (*A*) types of proteins, plus the fact that these will be oriented by surface forces in such regular manner that they can interlock. Molecular attraction forces among the two films would seal the surfaces to each other.[11] Furthermore, different degrees of adhesion might be explained by differences in the spacing of the molecules on either side of the phase boundary (*D*). Any change resulting in divergence of the biochemical characteristics or the physico-chemical parameters of the two systems in contact might upset either the orientation (*E*) or the compatibility (*F*) of these films and produce a release of one system from the other. This may likewise be attained by direct proteolytic action of enzymes present at the surface.

The association by surface contact between two nerve fibers, or even between a nerve fiber and a specifically impregnated non-nervous pathway, could then be viewed in the same light. The fact that we are seemingly dealing with a problem of very general biological applicability, may give renewed impetus to its analysis on the molecular level. So much about orientation and fasciculation of nerves.

The second problem to arise in this connection, as we have said, is one of numerical control. For the size of the fiber complement of a given nerve follows a definite norm, which raises the specific question of what determines the final size of a nerve? Four factors enter into consideration: Size of the nerve source; peripheral amplification by fiber branching; peripheral reduction by anastomosing; and the size of the periphery to be innervated. Each one of these factors contributes to the number of nerve fibers ultimately found in operation.

The size of a nerve source anticipates grossly the size relations of the future nerves; that is to say, the amount of neuroblasts disposed to send out peripheral nerve fibers varies in different parts of the central nervous system, in a certain forward reference to the later

[11]Compare the paper on *Protein Patterns in Cells* by F. O. Schmitt presented at this symposium.

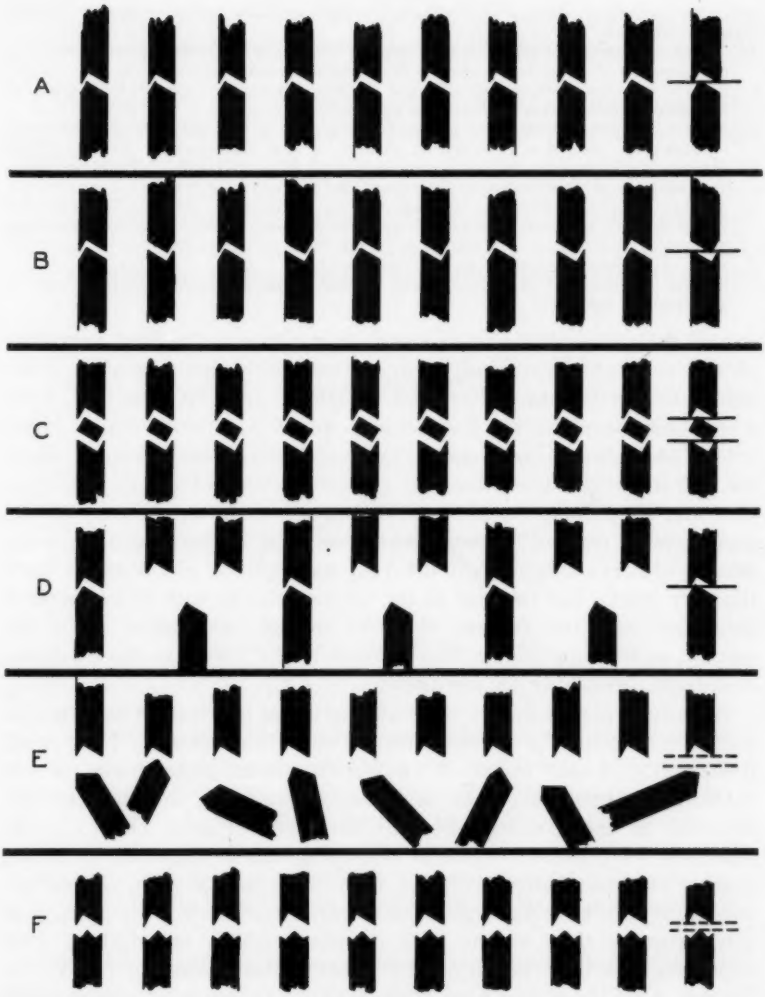

FIGURE 10

SCHEMATIC REPRESENTATION OF SELECTIVE ADHESION BETWEEN TWO ORGANIC SYSTEMS ON THE ASSUMPTION OF SPECIFIC PROTEIN CONFIGURATION

Each rod represents the polar end of a molecule. The notches and correspondingly shaped triangular protrusions symbolize complementary steric configurations in the molecules resulting in selective interlocking, according to the hypothetical analogy of antigen-antibody union. Vertical position of rods indicates parallel orientation of

molecules in surface films. Full lines on the right indicate surface adhesion, broken lines non-adhesion.

A, Surface application of one system to another containing molecules of complementary configuration (e.g., of nerve fiber to impregnated non-nervous pathway).

B, Surface adhesion between two identical systems, assumed to contain both types of mutually complementary molecules in identical ratios.

C, Surface adhesion between two identical systems, containing only one common type of molecule. Adhesion is mediated through a film consisting of an appropriate cementing substance (cf. Schmitt's presentation at this symposium). (Example: Selective adhesion of nerve fibers to their own kind.)

D, Weakened adhesion owing to different spacing (statistically speaking) of molecules in the apposed surfaces. The surface union in *D* would be only one third as strong as in *A*.

E, Lack of adhesion due to molecular disorientation.

F, Surface detachment, owing to change in the molecular configuration in one of the apposed surfaces.

size of the corresponding periphery. For instance, the limb segments of the cord are intrinsically larger than the trunk segments, even before the limbs have developed (Coghill, '36), and, in fact, even when limb development has been suppressed experimentally (Detwiler, '24). As we have said in the beginning, little is known about the factors which determine this typical spatial pattern of proliferation and differentiation and thereby produce the crude cast of the early central nervous system. Owing to these initial inequalities, the density of nerve outgrowth in different segments varies somewhat from the very start. But the size of the source and the rate of its increase determine only the amount of fibers leaving the centers, while the amount of terminal connections is much larger, owing to the extensive peripheral branching of the fibers.

Branching occurs in two ways: by terminal bifurcation and by collateral sprouting. As we have mentioned before, *terminal* bifurcation presumably results whenever two simultaneous pseudopodia of the growing tip turn out to be of equal strength, so that neither will succumb to the draining effect of the other (Figure 11, *A*). The tendency for profuse peripheral branching can be expected to be the greater the more intersections a fiber finds on its way. Therefore, branching will be much more extensive in a medium with unoriented ultrastructure than in one with definite micellar orientation. This expectation is fully borne out by tissue culture evidence (P. Weiss, '34, p. 440). According to Levi ('41), irradiating a culture with radium may greatly increase the incidence of branching, but the manner in which this occurs has not yet been analyzed. The rôle of the medium is likewise illustrated by the extensive branching which occurs in the unorganized scar around the cut end of a regenerating nerve. Perhaps the different density of the neropil in various parts

of the brain (Herrick, '34) will one day be correlated with the different density and micellar organization of the respective colloidal matrices.

Collateral branching, on the other hand, is the result of a new budding process from the consolidated stem of a fiber (Figure 11, *B*). Experiments of Peterfi and Kapel ('28) suggest that local mechanical irritation of the fiber can produce it. Speidel ('33) has shown that the agitation caused by a dividing cell on or near the nerve fiber may act similarly. Obviously, any irritant, which is sufficiently strong to remobilize the local fiber protoplasm may, if given further support

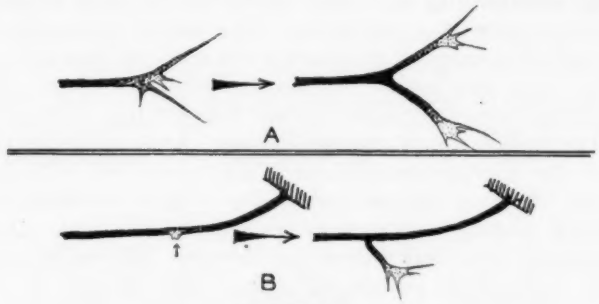

FIGURE 11

MODE OF BRANCHING IN NERVE FIBERS

A, Terminal branching, due to the persistence of two contemporary pseudopodia.
B, Collateral branching, due to lateral activation of stem of fiber.

by external conditions, lead to the establishment of a local outgrowth which then proceeds just like any other new fiber. The conditions under which this occurs remind us strongly of the rules established by Child ('27) for lateral regeneration in Hydroids; that is, in order to be effective, the wound stimulus must exceed a certain strength, and the distance from the existing apical end must be sufficiently great to insure to the new growth center what Child ('41) has called "physiological isolation."

Branching of peripheral nerve fibers occurs chiefly inside the terminal tissues. Each motor axon is eventually connected with a great number of muscle fibers through corresponding peripheral arborizations. There is evidence that muscle fibers are innervated long before they ever become muscle fibers, that is, in the state of myoblasts. This being the case, it is probable that whenever a myoblast or a young muscle fiber divides, this stimulus by itself provokes the formation of

a collateral branch from the nerve fiber, so that each new muscle fiber, as it emerges, takes its share of the neurone along. The fact that the average number of muscle fibers supplied by a single nerve fiber varies systematically for different muscles, would thus be an expression of the different rates of multiplication of the muscle fibers in different muscles.

The question of peripheral *anastomosing* of fibers by protoplasmic fusion is less clear. Boeke ('33) has emphasized the syncytial nature of the peripheral sympathetic plexus, and Stöhr ('35) has taken an even more extreme stand. Boeke ('30, '38) has also stressed the syncytial anastomosing of somatic nerves in the early phases of regeneration, a transitory condition which gradually gives way to fiber individualization from within the common mass. It seems that simple and thin fibers may merge peripherally, while the larger and more differentiated fibers always retain their individuality. This distinction is substantiated by nerve cultures in vitro. Levi ('41, p. 193) points out that the finest fibers frequently anastomose, while larger fibers never do. Might it not be that fusion is again contingent on full biochemical identity, which would exist among the primordial fibers, but disappear with their progressive divergent differentiation as they mature? This would explain the gradual individualization of fibers in regenerated nerves. However, these processes have not yet received enough attention to permit us to evaluate their significance for the final configuration of the peripheral nerve pattern.

Besides affecting nerve orientation and branching, the periphery regulates the volume of its innervation in two other ways. One of these has become familiar from the comprehensive studies of Detwiler and his co-workers (reviewed in Detwiler '36). Their experiments have shown that in urodele amphibians the size of the spinal ganglia is adjustable within limits to the actual extent of the periphery which they supply. An experimental increase or decrease of the peripheral tissue produces an augmentation or reduction in the number of spinal ganglion cells in the corresponding segments. May ('33) in the frog, and Hamburger ('34, '39) in the chick have demonstrated a similar relation between size of periphery and size of the motor nuclei of the cord. While the manner in which the expanse of the periphery reflects upon the volume of the centers is still wholly obscure, it is certain that the periphery exerts some influence on the number of fibers it is to receive right at their source.

Recently, however, we have come to learn about a second regulative

influence by the periphery which does not affect the production, but rather the admission of fibers, in that it determines what proportion of the total fiber production is actually admitted into final functional connections. It was noted that in the case of transplanted full-sized limbs innervated from a reduced nerve source (P. Weiss, '37), as well as in the case of undersized regenerating limbs innervated from a superabundant nerve source (Weiss and Walker, '34), the total amount of peripheral nerve branches bore a definite relation to the size of the limbs rather than to the size of the nerve sources. These experiments, amplified later by Litwiller ('38, '38*a*) have shown beyond doubt that the tissues of the limb exert a controlling influence on the admission of nerve fibers into the peripheral field. The experiments can be best summarized in a diagram (Figure 12). Faced with

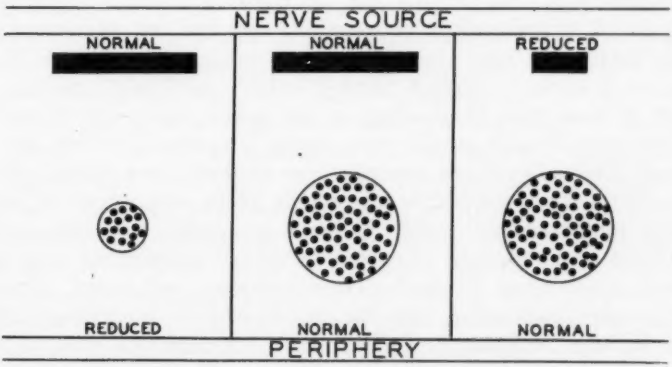

FIGURE 12

DIAGRAM, INDICATING LACK OF DEPENDENCE OF PERIPHERAL NERVE FILLING UPON SIZE OF THE NERVE SOURCE

an oversized nerve supply, the tissue can somehow prevent its becoming oversaturated with nerve fibers. Faced with an undersized nerve source, it can yet draw its full quota of peripheral branches. How it does this, is still unknown. A direct experimental demonstration of the effect has thus far been furnished only for regenerated nerves. However, the remark by Harrison ('35, p. 148), that a giant limb grown on a small body may contain a motor nerve complement proportional to its size without corresponding increase of the innervating centers, indicates that a similar mechanism of control operates in the embryo.

From these studies it would seem, that an innervated area spreads some influence which prevents the penetration of further innervation into its domain. It stakes off a territory. This may account for a well known observation. When a skin nerve is cut, the denervated area is slowly invaded by collateral nerve fibers from surrounding intact areas: the denervation has apparently removed some restraining influence previously exerted by the former occupants of the territory.

Thus receptor and effector tissues regulate the density of their innervation. The mechanism need not be the same for all tissues, and each one will have to be accorded a separate investigation. In only one case do we have more concrete information, that of the muscle. It is a well established anatomical fact that the large majority of muscle fibers receive but one single motor nerve branch each. Harrison ('10) has pointed to the analogy between this fact and the monospermy of eggs. Just as an egg, after receiving a single spermatozoan, would produce a surface reaction through which additional spermatozoa would be kept from penetrating, so the muscle fiber, after admitting its first nerve branch, would have become immune to further impregnation. This subject has recently been studied more extensively by Fort ('40). It was confirmed that muscle fibers, even when confronted with a superabundant supply of nerve terminals, as a rule, cannot be forced or conditioned to accept functional connections with more than a single nerve terminal. There are some indications, although not yet very convincing, that the reason why an innervated muscle fiber becomes resistant to further nerve impregnation may lie in a change of its surface constitution. The muscle, however, is the only case in which at least a beginning has been made to analyze the nature of what we may call the "saturation factor" of peripheral innervation.

Lack of space as well as of reliable information prevents us from going into the subject of nerve fiber *size*, the diameter of the individual fiber. One observes fibers in all gradations, from down at the limits of microscopic visibility up to those visible to the naked eye. They can be grouped into different size classes with different physiological properties (Erlanger and Gasser, '37), and different nerves contain fibers of the different classes in different proportions. These proportions seem to be essentially re-established in regeneration (P. Weiss, '37, p. 517), and the question of what determines the diameter of a nerve fiber seems to offer considerable interest. However, I know of

no single analytical examination devoted to this problem. Correlations have been established from observational data between the length of fibers and their size, between innervated area and size, and the like, but with the possible exception of observations by Detwiler and Lewis ('25), indicating that motor neurones may show size reduction in the case of an experimental reduction of their periphery, no analytical experiments are available to decide in which way intrinsic and environmental factors contribute to the determination of the final dimensions of a neurone. We may mention that in tissue culture nerve cells and fibers occur in all sizes, but their variation is too unsystematic to furnish any clues.

Let me stop here with the analytical part of the discussion. The picture I have given you has covered only the most prominent features of nerve patterns. If I were to be exhaustive, I would have to go on and discuss the finer differentiation and physiological distinction among fibers, the arborization of the dendrites in the gray, the problem of the stratification of the brain, the problem of the relation between nerve fibers and capillaries, the differential susceptibility to hormonal agents which may be of behavioral significance, the causes of plexus formation, the association with sheath cells, the production of myelin, and many other features. But even the very incomplete picture, which you have received, will have impressed you with the multiplicity and complexity of factors which participate in turning out the final intricate fabric as which the nervous system confronts the anatomist and physiologist. If, in spite of this multiplicity and diversity of agents, the end product turns out to be, on the average, a nervous system fully capable of coördinating, controlling, and integrating the functions of the body, the credit for this achievement must be ascribed to three main factors.

Firstly, to the existence in the embryo of a definite spatial and chronological organization, according to which the individual events are seriated. Secondly, to the fact that the initial organization provides only for the gross outlines of the future development, leaving sufficient latitude for direct adjustive interactions to allow for a certain amount of variation among individuals. And thirdly, to the fact that the operation of the nervous system does not require absolute structural stereotypism, so that only statistically speaking must there be something like constancy and repetition in the final developmental product.

Just a few words in amplification of these three principles. The

standardized dosing and timing of the morphogenetic processes is something the nervous system has in common with other parts of the body, and need not be discussed here (cf. P. Weiss, '39, pp. 104, 319, 486, 558).

The second point, however, deserves illustration because it gives a tangible conception of what we usually refer to as "regulation." Let us take a specific example. Remember that the formation of a straight nerve connection between a center and a peripheral organ is due to a dual process: Oriented outgrowth of the pathfinders, and the subsequent filling up of the cable by selective fasciculation. Suppose now, that owing to a genetic mutation or to developmental accidents, the pioneering fibers grew out precociously, before their supposed leading structure is ready: they will stray about and take devious routes, but some of them have still a good chance to reach the periphery. After that, selective fasciculation will do its part and produce a fair-sized nerve connection after all. The initial size deficit of the nerve may be partly corrected by the stimulative influence of the periphery which will mobilize additional neurones. But even with a constantly undersized nerve source, the peripheral field can still obtain its full quota of innervation, owing to the control it exerts over the amount of peripheral branching. The fact that each neurone will now have to carry a heavier load, is immaterial, because the size of the innervating centers is no measure of functional perfection, as is illustrated by the perfect functioning of transplanted supernumerary limbs innervated from only a fraction of the normal number of motor neurones (P. Weiss, '36). We realize, thus, that quite a few steps in the complex process of nerve formation may deviate from the norm without endangering the essential adequacy of the final product.

This situation again is characteristic not only of the development of the nervous system, but of development in general. One must remember that no developmental process is a unitary event. Innumerable independent and interdependent partial events share in the formation of an embryonic part. So, if one among the many fails to coöperate properly, the result may be only slightly off normal. This is merely translating the conclusions at which the geneticist has arrived in his multiple factor analysis from the symbolic language of genetics into the concrete terms of embryodynamics (cf. P. Weiss, '39, p. 479-486).

The latitude left to developmental processes leads us to the third point. No two nervous systems are identical or even nearly identical.

if we concentrate on details. Yet, many of the most popular schemes to interpret the physiological action of the nervous system in terms of neurone relations, are based on the assumption that every detail of design and measurement in each individual neurone is significant as such. The number of collaterals, the length of collaterals, the points where they branch off, the number of end feet, their density on the cell body, the spacing between individual endings—all such details are treated as if there were agents in the organism which could wisely see to it that each individual neurone would really be as precisely constructed as physiological theory demands.

Now, while we are still far from complete insight into the factors which operate in establishing the finer structural details of the neurone architecture—and there is no saying what surprises the future may hold—we do know certain things; and one of them is that those factors which we have discussed in this report and which certainly do operate, are not of the kind that could produce the envisaged precision machine. The finished machine can be of no greater precision than the agents by which it has been constructed. We do not mean to deny here the possible intimate correlation between structural aspects and physiological performance altogether, but we must point out that, if the neurophysiologist wants to take into account the lessons of embryology, he will have to substitute *statistical* considerations for his overemphasis on *systematic* traits.

For instance, the *average* density of dendrites or collaterals in a certain neurone group may be functionally relevant, even though the number of dendrites or collaterals on each element may fluctuate considerably about that average number. As we have said before, the statistical amount of branching is a function of the colloidal consistency of the medium, and could, therefore, be very well understood on embryological principles. On the other hand, the assumption that each and every nerve cell of the group might possess a fixed number of dendrites or collaterals is positively discouraged by what we know about developmental mechanisms.

Here is another example of how statistical properties can play as good an explanatory rôle as is usually ascribed to structural details. The phenomenon of central irradiation, first described by Sherrington, implies that, as the excitatory influx into a center increases, more and more cells become engaged, leading to a proportional increase of the response. This is usually explained on the assumption that the central cells are connected in definite chain arrangements. In

spreading from one cell to the next, synaptic resistance must be overcome. Thus, the stronger the initial charge of the first excited cell, the more hurdles it will be able to take, and the more cells of the chain will be set off. But since each cell is viewed as a link in several intersecting chains, the structural provisions prerequisite for an orderly gradation of the response would have to be infinitely complicated. In contrast to this view, I have recently obtained evidence of graded responses, increasing with the strength of the stimulus, in deplanted centers (see p. 179), in which every trace of systematic organization had vanished. In these deranged neurone pools, cells are still interconnected, but at random. If graded responses can still be obtained, there seems to be only one explanation: That is, that central cells develop as a population in which the *thresholds* of excitability vary *at random* (P. Weiss, '41). In such a population the number of elements of a given threshold class, plotted against the thresholds, would follow a near-normal distribution curve (Figure 13, *A*). A stimulus of given

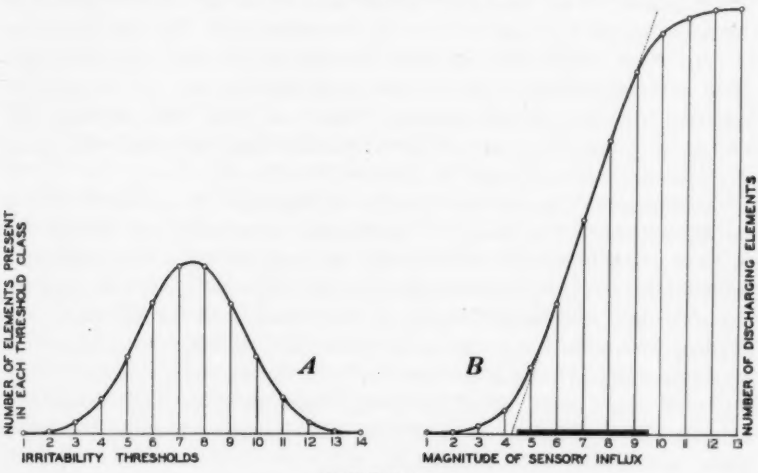
NUMBER OF ELEMENTS PRESENT IN EACH THRESHOLD CLASS
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
IRRITABILITY THRESHOLDS
B
1 2 3 4 5 6 7 8 9 10 11 12 13
MAGNITUDE OF SENSORY INFLUX
NUMBER OF DISCHARGING ELEMENTS

FIGURE 13

HYPOTHETICAL DISTRIBUTION CURVE OF THRESHOLDS OF EXCITATION IN A POPULATION OF NERVE CELLS

(Explanation in text.)

strength would set into action all cells of the corresponding and lower thresholds. The number of cells activated by stimuli of varying strength would be expressed by the integral of the normal distribution

curve, and this, a sigmoid curve, very nearly approaches the course of a straight line for about 80 per cent of its range (Figure 13, *B*). Thus, within that range, any increase of the stimulus would bring in a proportionally larger number of cells, without the need for any special structural provisions. It is interesting to note, that v. Brücke, Early, and Forbes have actually demonstrated that the distribution of thresholds among the α fibers in a peripheral nerve follows a normal distribution curve, in other words, expresses random fluctuation within a population of living elements, rather than any systematic provisions specially devised in development. I think Hecht ('26), in his treatment of the visual apparatus, was the first to suggest such statistical considerations.

It has been my intention in this review not only to point out the multiplicity and complexity of factors that enter into the making of a nervous system—and of any organic system for that matter—but also to indicate the analytical insight that may be gained by resolving the complex situations into their simpler components and treating these one by one. In doing this, we have seen that the resolution can sometimes even be carried to the point where the biophysicist proper may take over. Once a vital process has been reduced to terms of molecular events, nothing remains to be done by the biologist in that particular direction. Thus, after having deposited the problems of adhesivity, selectivity, ultrastructure, etc., at the doorstep of the biophysicist, we may withdraw expectantly to see what further elucidation he may be able to provide from his own province. Or better still, we may combine forces with the biophysicists to create teamwork as proficient as that of that well-known symbiosis of the fable, the blind and the lame.

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